Biochemical Pharmacology 83 (2012) 1383–1392

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Double-edged sword effect of biochanin to inhibit nuclear factor kappaB: Suppression of serine/threonine and tyrosine kinases

Sunil Kumar Manna *

Laboratory of Immunology, Centre for DNA Fingerprinting & Diagnostics, Nampally, Hyderabad 500 001, India

ARTICLE INFO

Article history:
Received 9 November 2011
Accepted 15 February 2012
Available online 22 February 2012

Keywords:
Protein tyrosine kinase
Ser/Thr kinase
Biochanin
NF-κB
IKK
PTK inhibitors

ABSTRACT

Several protein tyrosine kinase (PTK) inhibitors predominantly isoflavones, such as genistein, erbstatin, quercetin, daidzein, present in red clover, cabbage and alfalfa, show apoptotic effect against cancer cells. In this study I found that biochanin, a methoxy form of genistein, inhibits IL-8-mediated activation of nuclear transcription factor kappaB (NF-κB) and activator protein 1 (AP-1) more potently than genistein as shown in Jurkat T-cell line. Both biochanin and genistein potently inhibited activity of Lck and Syk, but biochanin specifically inhibited activity of IKK. Biochanin inhibited completely NF-κB activation induced by PMA, LPS, pervanadate (PV), or H_2O_2, but only partially that induced by TNFα. Genistein was unable to inhibit IL-8-induced IKK activity, but it blocked PV-induced IKK activity. Biochanin inhibited activation of NF-κB by TRAF6 completely, but by TRAF2 partially. *In silico* data suggested that biochanin interacted strongly with serine/threonine kinase than genistein, though both equally interacted with PTK. The data show that both biochanin and genistein are potent inhibitors of PTK, but biochanin is a potent inhibitor of serine/threonine kinase too. Formononetin, having hydroxyl methoxy group is less potent to inhibit IKK than biochanin. Biochanin inhibits NF-κB activation not only by blocking the upstream IKK, but also PTK that phosphorylate tyrosine residues of IκBα. Thus, the double-edged sword effect of inhibition of NF-κB *via* inhibition of both serine/threonine kinase and PTK by biochanin might show useful therapeutic value against activities of cells that lead to tumorigenesis and inflammation.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Protein tyrosine kinases (PTKs) play a crucial role in many cell regulatory processes. Functional perturbation of PTKs results in many diseases. PTKs are mostly activated by the growth factors and cytokines through interaction with their specific receptors. Autophosphorylation of these receptors leads to recruitment of several PTKs and thereby activate cell signaling and cell cycle progression [1]. In tumorigenesis several fold activation of these kinases occurs often initiating alarm signal for its regulation. Upon stimulation by cytokines, microbial agents such as bacterial and fungal toxins and viral proteins and DNA, oncogenes, chemotherapeutic agents, and environmental stress, several serine/threonine kinases (Ser/Thr kinases) participate in activation of various transcription factors including nuclear transcription factor kappaB (NF-κB), activator protein 1 (AP-1), cyclic AMP-responsible element binding factor (CREB), *etc.* [2]. The dual kinase inhibitors often help in developing suitable therapeutic molecules against tumorigenesis. Activation of tyrosine and/or Ser/Thr kinases often induces cells for rapid proliferation that lead to tumorigenesis. Interleukin-8 (IL-8), a chemokine and an inducer of angiogenesis, induces cells after interaction with its specific seven transmembrane containing receptors, IL-8Rs [3]. These activate Ser/Thr kinases like mitogen activated protein kinases (MAPKs) through induction of small GTPase and IκBα kinase (IKK) *via* recruitment of TNF receptor associated factor (TRAF)6 [4]. *Clostridium botulinum* C3 transferase (C3-toxin), an inhibitor of Rho-GTPase is shown to inhibit MAPKs [5]. Several inducers like phorbol myristate acetate (PMA), lipopolysaccharide (LPS), TNFα are activators of Ser/Thr kinases and some of them phosphorylate Tyr^{42} of IκBα possibly activating IKK complex [6]. H_2O_2 and pervanadate (PV) activate tyrosine kinases, like Syk [7]. TNFα induces Ser/Thr kinases like MEKK, IKKs, NIK, *etc.* by recruiting TRAF2 and 6 [8]. Epidermal growth factor (EGF) induces cells through binding of EGF receptor, a typical receptor tyrosine kinase (RTK), followed by activating downstream MAPKs [9].

Abbreviations: AP-1, activator protein 1; C3-toxin, *Clostridium botulinum* C3 transferase; CE, cytoplasmic extracts; Cox2, cyclooxygenase 2; ICAM, intercellular adhesion molecule; NF-κB, nuclear transcription factor kappaB; IL, interleukin; IκB, inhibitory subunit of NF-κB; IκBα-DN, IκBα dominant negative; IKK, IκB kinase; Lck, lymphocyte-specific PTK (protein tyrosine kinase); LPS, lipopolysaccharide; NE, nuclear extracts; PV, pervanadate; RMSD, root mean square deviation; Syk, spleen tyrosine kinase; TNFα, tumor necrosis factor alpha; TRAF, TNF receptor-associated factor.

* Tel.: +91 40 24749412; fax: +91 40 24749448.
E-mail address: manna@cdfd.org.in.

0006-2952/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2012.02.011

Phytoestrogens possess anti-inflammatory, anti-allergic, anti-oxidant, anti-thrombotic, anti-neoplastic, and hepatoprotective activities [10]. Red clover dietary supplements contain varying ratios of the four isoflavones commonly found in legume-based diets, namely, daidzein, genistein, formononetin, and biochanin. Several phytoestrogens are isoflavonoids and are inhibitors of PTK. Genistein is a trihydroxyisoflavone that inhibits PTK by competing with ATP [11]. Like genistein, biochanin is known to inhibit PTK and to interfere cell growth [12,13]. Biochanin is a dihydroxy methoxyisoflavone. Erbstatin, a dihydroxymethylcinnamate, inhibits PTK by competing with ATP and peptide substrate at the catalytic site [14]. Daidzein, a dihydroxyisoflavone and a structural analog of genistein, lacks PTK inhibitory activity. Quercetin, a pentahydroxyisoflavone inhibits PTK at lesser extent. Formononetin, a hydroxymethoxyisoflavone in food materials, is absorbed as daidzein in the gut and shows PTK inhibitory activity. Formononetin is also increased the allergic responses by increasing the amount of IL-4 [15] and induces cell death [16]. Several isoflavonoids, including biochanin, have been shown to inhibit growth and induce apoptosis in bladder, prostate, and breast cancer cells [17–19].

This study examined the ability of these isoflavones found in red clover to inhibit inflammatory and proliferative activities of cells. Isoflavones have shown to reduce the synthesis of prostaglandin E2 and/or thromboxane B2 by inhibiting cyclooxygenase 2 (cox2) [20]. However, the mechanism of anti-proliferation and anti-inflammatory activity of biochanin has not been understood. Nuclear transcription factor kappaB is a rate-limiting transcription factor underlying both inflammatory and proliferative activities of cells. NF-κB activation depends upon the translocation of homo- or hetero-dimers of Rel family proteins from cytoplasm to nucleus. Dimer of Rel family proteins are often arrested in the cytoplasm by inhibitory subunit of kappaB (IκB) family proteins. Upon stimulation, IκB family proteins are phosphorylated followed by ubiquitinated and degraded. The IκB family proteins are phosphorylated by IκBα kinases, predominantly a Ser/Thr kinase. The Tyr^{42} residue of IκBα is also phosphorylated by unknown tyrosine kinase and these are activated by H_2O_2 and PV [6]. We have shown previously that biochanin exhibits anti-proliferative, antiinflammatory activities by inhibiting NF-κB [21]. The present study shows that biochanin is a potent inhibitor of not only PTK, like other isoflavones, but also inhibits Ser/Thr kinases especially IKK complex. First time, I am providing data that methoxy genistein is a potent inhibitor of Ser/Thr kinase. Thus, biochanin is a dual kinase inhibitor and showed double-edged sword effect to inhibit NF-κB and may be use as a potent anti-inflammatory and/or anti-tumor agent.

2. Materials and methods

2.1. Materials

Biochanin, quercetin, genistein, formononetin, daidzein, quercetin, phorbol myristate acetate, doxorubicin, lipopolysaccharide, MTT, DMSO, H_2O_2, sodium orthovanadate, and anti-tubulin antibody were obtained from Sigma–Aldrich Chemicals (St Louis, MO, USA). Erbstatin was obtained from LC Laboratories (Woburn, MA, USA). Penicillin, streptomycin, neomycin, RPMI 1640, DMEM medium, and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY, USA). Antibodies against ICAM1, cox2, IκBα, p65, p50, CRM1, IKKα, IKKβ, Lck, Syk, and phospho-Syk and gel shift oligonucleotides for NF-κB and AP-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant IL-8, EGF, and TNFα was purchased from Peprotech (Rocky Hill, NJ, USA). *Clostridium botulinum* toxin (C3-toxin) was kindly provided by Prof. G.S. Chhatwal (German Center for Biotechnology, Braunschweig, Germany). Plasmid constructs for *TRAF2*, *TRAF6*, *p65*, *IκBα-DN*, *Cox2-luciferase*, and *NF-κB-luciferase* were obtained from Prof. Bharat B. Aggarwal (MD Anderson Cancer Center, Houston, USA).

PV was prepared freshly for treatment of cells as described before [6]. Briefly, 20 μl of 1 M sodium orthovanadate was taken in 270 μl phosphate-buffered saline. Ten microliters of 33% H_2O_2 was added to the mixture and reaction was continued for 5 min at room temperature. The pH of the solution was neutralized by adding 1N HCl and excess H_2O_2 was deactivated with catalase (60 μg). The concentration of PV generated is denoted by the vanadate concentration taken in the reaction mixture. The vehicle control was used the mixture without sodium orthovanadate for treatment.

2.2. Cell line

The cell line used in this study Jurkat (human T-cells) was obtained from American Type culture collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were free from mycoplasma, as detected by Gen-Probe mycoplasma rapid detection kit (Fisher Scientific, Pittsburgh, PA, USA).

2.3. NF-κB and AP-1 DNA binding assay

DNA binding of AP-1 and NF-κB was determined by gel shift assay [22]. Briefly, the cells were subjected to different treatments as described and cytoplasmic and nuclear extracts were prepared. Nuclear extract (8 μg protein) was incubated with ^{32}P-end-labeled double-stranded NF-κB oligonucleotide of HIV-LTR, 5′-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3′ for 30 min at 37 °C, and the DNA–protein complex was separated from free oligonucleotides on 6.6% native PAGE. AP-1 DNA binding was assayed similarly using specific double-stranded labeled oligonucleotides. Visualization of radioactive bands was done in a fluorescent image analyzer FLA-3000 (Fuji, Japan).

2.4. NF-κB-, AP-1-, and cox2-dependent reporter gene transcription assay

The amount of different transcription factor-dependent reporter gene, luciferase expression was carried out as described previously [23]. Jurkat cells were transfected with *NF-κB-luciferase*, *AP-1-luciferase*, or *Cox2-lucifearse* (0.5 μg) and GFP (0.5 μg) constructs. After 3 h of transfection cells were washed and cultured for 12 h. GFP positive cells were counted (35–40% for different combinations). Cells, after different treatments were extracted with lysis buffer (part of Luciferase assay kit from Promega) and luciferase activity was determined and indicated as fold activation considering vector-transfected value as 1 fold.

2.5. IKK and Lck activities assay

The IKK and Lck activities were assayed by a method described previously [23,24]. Briefly, IKK or Lck complex from whole-cell extract (300 μg) was precipitated with anti-IKKα and -IKKβ antibodies (1 μg each) or anti-Lck antibody (1 μg), followed by incubation with protein A/G-Sepharose beads (Pierce, Rockford, IL, USA). After 2 h incubation, the beads were washed with lysis buffer and then assayed for IKK by using 2 μg of substrate GST-IκBα (aa1–aa54) or Lck activity was assayed by autophosphorylation of Lck using assay buffer with ^{32}P-γATP.

2.6. Study of molecular docking

2.6.1. Proteins setup for docking

Experimental structures of serine/threonine kinase (PDB ID: 2QON) [25] and tyrosine kinase (PDB ID: 3GQL) [26] were retrieved

from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) [27]. Serine/threonine kinase has single A chain and pdb file was edited by removing hetero atoms such as glycerol and water molecules, whereas, tyrosine kinase has three identical subunits – A, B, and C. The study was carried out on only the A subunit of the enzyme and other heteroatom's chains are removed. In order to use the protein in the AutoDock docking simulation program, all polar hydrogen were added to both serine/threonine and tyrosine kinase with the GROMACS modeling package [28]. The structures were optimized in 400 steps of conjugate gradient minimization, employing the GROMACS87 force field. During minimization, the heavy atoms were kept fix at their initial crystal coordinates, but added hydrogens were made free to move. Minimization was effected under a vacuum medium. Electrostatic interactions were calculated using the cut-off method. As the acceptable minimal force gradient was reached, the minimization converged and the resultant structures were saved. Finally, solvation parameters were added using the ADDSOL utility of AutoDock 4.0.

2.6.2. Ligands setup

Biochanin (CID: 5280373) and genistein (CID: 5280961) were loaded from PUBCHEM database. Hydrogen atoms were added to each ligand and ionization states were determined considering a pH of 7.0 using Prodrg Server [29]. The GROMACS package (fast energy minimization of the ligands by GROMACS force field) and the GAMESS package (complete energy minimization by AM1 semi-empirical method, calculation of partial charges) are applied on two ligands [30].

2.6.3. Docking protocol

All the docking clusters are performed on a Linux Platform (Intel Xeon 5450 Quad Core 3.0 GHz,) All the docking simulations were performed using AutoDock4.0 [31]. Each docking experiment was performed 50 times (100 million energy evaluations each), yielding 50 docked conformations. Parameters for the docking are as follows: population size of 150; random starting position and conformation; maximal mutation of 2 Å in translation and 50° in rotations; elitism of 1; mutation rate of 0.02 and crossover rate of 0.8; and local search rate of 0.06. Simulations were performed with a maximum of 2.7 million energy evaluations and a maximum of 27,000 generations. Cubic grid, centered on the active site with 26.25 Å × 18.75 Å × 26.25 Å dimensions and grid spaced by 0.375 Å was generated using AutoGrid4 for each structure.

2.7. Statistical analysis

Results were expressed as mean ± SD of three independent experiments. Statistical analyses of the samples were done by student's *t*-test wherever applicable. The $p < 0.05$ was considered to be significant.

3. Results

No significant increase in the activity of lactate dehydrogenase (LDH) was observed upon treatment of cells with different concentrations of biochanin and genistein till 100 μM for 48 h by measuring lactate dehydrogenase activity from culture supernatant indicating lack of cytolysis. All the PTK inhibitors used in this study (Fig. 1A) were dissolved in DMSO at 10 mM concentration. Further dilution was carried out in the medium. The DMSO and diluents (referred as vehicle) had no effect on activation of NF-κB on cells alone or in IL-8-induced condition (Fig. 1C, left lanes).

3.1. Biochanin, but not erbstatin, genistein, quercetin, or daidzein inhibits NF-κB DNA binding

To determine the role of PTK inhibitors on NF-κB activation, Jurkat cells, treated with 10 μM of biochanin, erbstatin, genistein, quercetin, and daidzein for 6 h were stimulated with 100 ng/ml IL-8 for 3 h. Nuclear extracts (NE) were prepared and NF-κB DNA binding was assayed by gel shift assay. These PTK inhibitors had no effect on NF-κB DNA binding, but only biochanin-pretreated cells showed inhibition IL-8-induced NF-κB DNA binding (Fig. 1B). This result suggests that biochanin might be potent inhibitor to block IL-8-induced NF-κB activation.

3.2. Biochanin suppresses NF-κB DNA binding and NF-κB-dependent gene expression

IL-8 increased NF-κB DNA with increasing concentrations and biochanin, not genistein almost completely inhibited IL-8-induced NF-κB DNA binding (Fig. 1C). To understand the role of biochanin on inflammatory responses, I have used IL-8 as an inducer of inflammation and, to begin with, adhesion molecules and cox2 were detected as inflammatory mediators. In Jurkat cells, IL-8 potently increased the amounts of cox2 and ICAM1 kinetically and biochanin-pretreated cells showed complete inhibition of these molecules (Fig. 1D). The amount of tubulin remained equal in these blots suggesting the loading control. The activity of luciferase induced by IL-8 kinetically in *NF-κB-luciferase* or *Cox2-luciferase* construct transfected cells was completely inhibited by biochanin (Fig. 1E).

Biochanin inhibited IL-8-induced IκBα degradation and p65 nuclear translocation as shown by the gradual decrease of p65 in cytoplasmic extracts (CE) obtained from IL-8 stimulated cells. Biochanin pretreated cells showed complete restoration of p65 at any time of IL-8 stimulation (Fig. 1F, upper panel). IL-8-stimulated cells' NE showed gradual increase in the amount of p65 with time of incubation and biochanin pretreated cells showed complete inhibition of this increase of p65 (Fig. 1F, lower panels). The amount of p50 was observed equally in both CE and NE at any time of IL-8 stimulation in presence or absence of biochanin. When IL-8-induced NE were incubated with different concentrations of biochanin, the NF-κB DNA binding did not altered *in vitro* (Fig. 1G). This suggests that biochanin-mediated inhibition of NF-κB activation requires cellular mechanism.

3.3. Biochanin, but not genistein inhibits IL-8-induced NF-κB, AP-1, and IKK activation

To compare the non-methoxy *versus* methoxy form of isoflavonoids on regulation of inflammation, I have studied the effects of biochanin and genistein on IL-8-mediated NF-κB and AP-1 activation. Biochanin or genistein alone had no effect on NF-κB or AP-1 DNA binding. Biochanin inhibited IL-8-induced NF-κB and AP-1 DNA binding (Fig. 2A1and C1), but not genistein (Fig. 2A2 and C2). IL-8 induced 8.6 fold ($p < 0.001$) or 11 fold ($p < 0.05$) NF-κB-dependent (Fig. 2B1) or AP-1-dependent (Fig. 2D1) luciferase activity in *NF-κB-luciferase* or *AP-1-luciferase* transfected cells respectively Biochanin inhibited IL-8-induced luciferase activity in a dose-dependent manner in those transfected cells. Genistein inhibited IL-8-induced luciferase activity marginally mediated by NF-κB (Fig. 2B2) or AP-1 (Fig. 2D2). These data further support that methoxy form of genistein *i.e.* biochanin is potent to inhibit NF-κB and AP-1 activation.

As NF-κB acts *via* activation of IKK complex, I have tested the activity of IKKs upon treatment of cells with several PTK inhibitors. I have noticed that only biochanin inhibited IL-8-induced NF-κB DNA binding, and only biochanin, but not genistein, erbstatin,

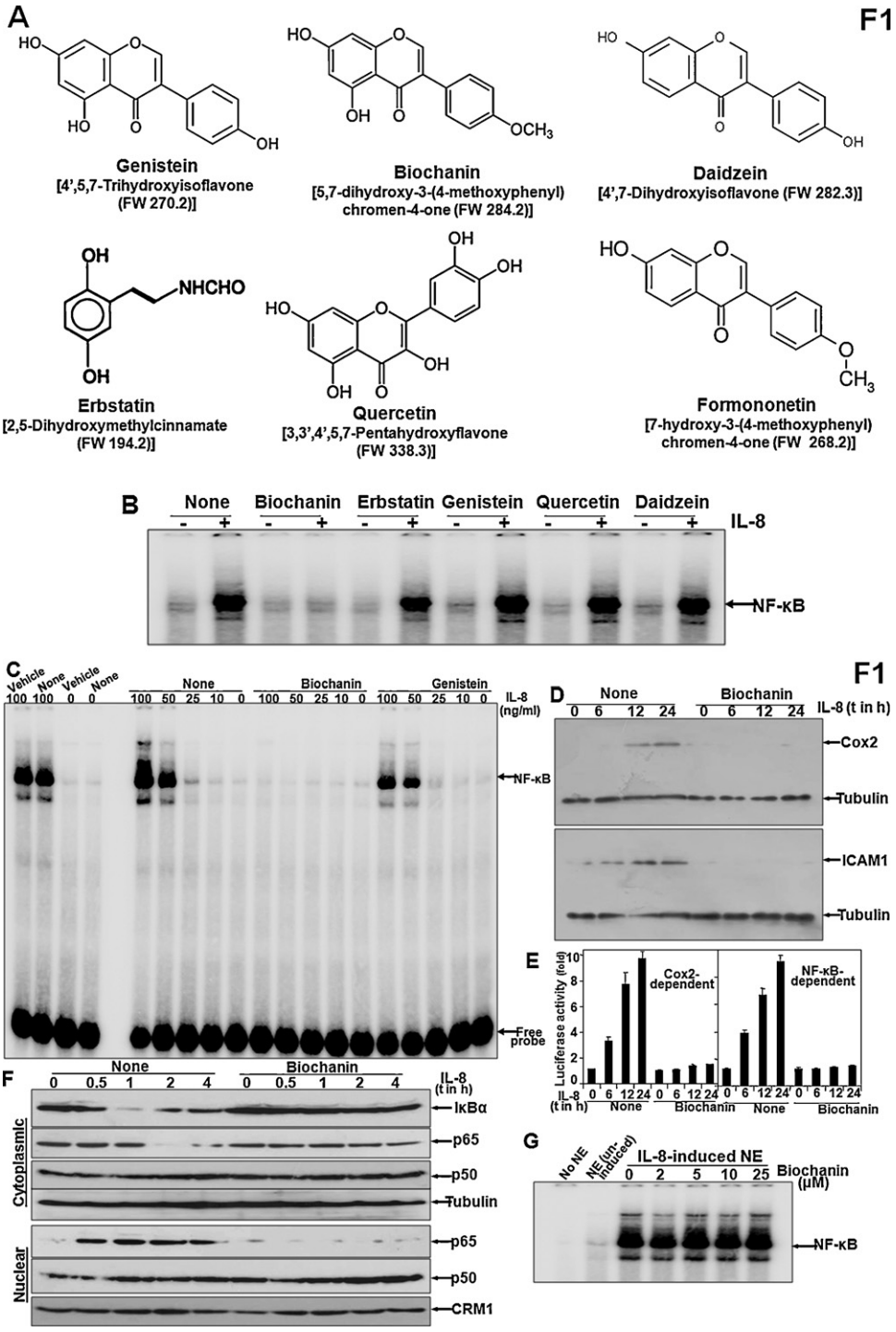

Fig. 1. Effect of PTK inhibitors on NF-κB activation. Structure of genistein, biochanin, daidzein, erbstatin, quercetin and formononetin are indicated (A). Jurkat cells were pretreated with 10 μM of biochanin, genistein, quercetin, or daidzein for 6 h and then stimulated with 100 ng/ml for 3 h. NE were prepared and NF-κB DNA binding was assayed by gel shift assay (B). Cells were treated with 10 μM of biochanin or genistein for 6 h and then stimulated with different concentrations of IL-8 for 12 h. Cells were treated with 2.5 μl DMSO (Vehicle) and then stimulated with 100 ng/ml IL-8 for 12 h. NE were prepared and measured for NF-κB DNA binding (C). Jurkat cells (2×10^6/ml) were treated with 10 μM biochanin for 6 h and then stimulated with 100 ng/ml IL-8 for different times. Whole cell extracts (100 μg proteins) were prepared and the amounts of cox2 and ICAM1 were measured by Western blot (D). The amount of tubulin was measured in these blots by co-incubating monoclonal anti-tubulin Ab. Jurkat cells (1×10^6/ml), transfected with Qiagen superfect transfection reagent for 3 h with plasmids *Cox2-luciferase* or *NF-κB-luciferase* (0.5 μg/sample) and *GFP* (0.5 μg/sample) constructs and cultured for 12 h. The GFP positive cells were counted under fluorescence microscope and these were 40–45% for different transfection conditions, indicating the transfection efficiency. Cells were then treated with 10 μM biochanin for 6 h followed by stimulated with 100 ng/ml IL-8 for different times. Cell pellet was extracted and assayed for luciferase activity expressed by cox2 and NF-κB (E). The experiments were repeated 4 times and results are represented as fold of activation over the nontransfected control. Jurkat cells either untreated or treated with biochanin (10 μM) for 6 h were stimulated with IL-8 (100 ng/ml) for different times. The amount of IκBα, p65, and p50 were measured from CE and p65 and p50 was measured from NE by Western blot (F). IL-8-induced Jurkat cells' NE were incubated with different concentrations of biochanin for 2 h at 37 °C and then NF-κB DNA binding was assayed (G).

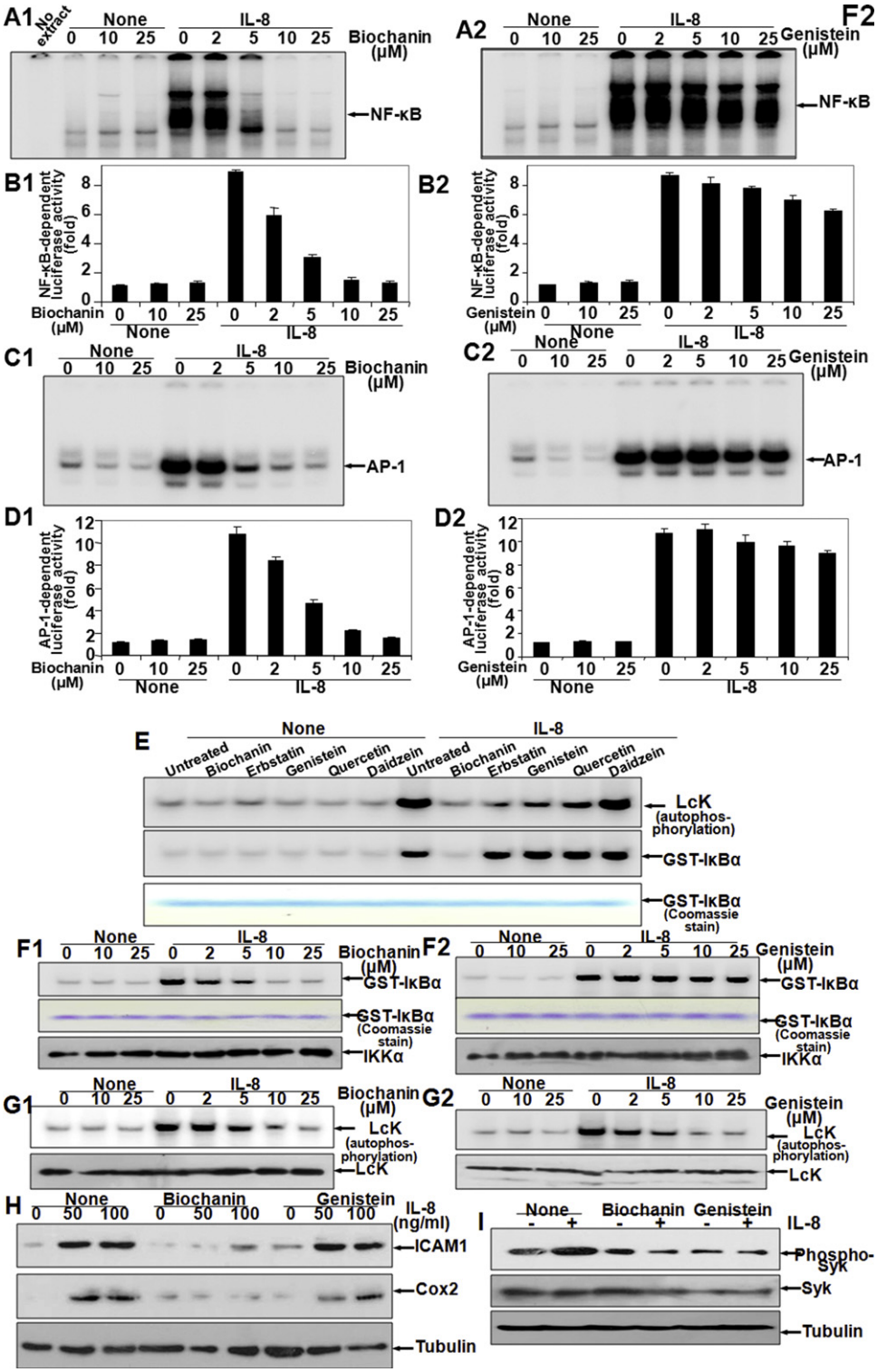

Fig. 2. Effect of biochanin and genistein on IL-8-induced activation of NF-κB, AP-1, IKK, and Lck. Jurkat cells were transfected with plasmids for *NF-κB-luciferase* or *AP-1-luciferase* and *GFP*. After washing, cells were cultured for 12 h. The GFP positive cells were counted and transfection efficiency was calculated. Cells were then treated with different concentrations of biochanin or genistein for 6 h followed by IL-8 (100 ng/ml) for 12 h. Nuclear extracts were prepared, NF-κB (A1 and A2) and AP-1 (C1 and C2) DNA binding was measured by gel shift assay. Cell pellet was extracted and assayed for luciferase activity in *NF-κB-luciferase* transfected cells upon biochanin or genistein treatment (B1 and B2) or *AP-1-luciferase* transfected cells upon biochanin or genistein treatment (D1 and D2). Jurkat cells were treated with 10 μM of biochanin, erbstatin, genistein, quercetin, or daidzein for 6 h and then stimulated with IL-8 (100 ng/ml) for 6 h. The whole cell extracts (300 μg protein) were immunoprecipitated with anti-IKKα and IKKβ (1 μg each) Abs and kinase was assayed using GST-IκBα as substrate (E, lower panel). Similarly, proteins were immunoprecipitated with anti-Lck (1 μg) Ab and kinase was assayed using ^{32}P-γATP in the assay buffer and the autophosphorylation of pull-down Lck protein (E, upper panel). Cells were treated with different concentrations of biochanin or genistein for 6 h and then stimulated with IL-8 (100 ng/ml) for 6 h. The whole cell extracts were used to assay IKK activity using GST-IκBα as substrate (F1 and F2). The Coomassie-stained gel was indicated in the middle panel. The IKKα measured from 100 μg protein extract by Western blot. Similarly, proteins were immunoprecipitated with anti-Lck (1 μg) Ab and kinase was assayed (G1and G2). The Lck was measured from 100 μg protein extract by Western blot. Jurkat cells, treated with 10 μM of biochanin or genistein for 6 h were stimulated with 50 and 100 ng/ml IL-8 for 6 h. The amounts of cox2 and ICAM1 were determined by Western blot (H). Jurkat cells were treated with 10 μM of biochanin or genistein for 6 h and then stimulated with IL-8 (100 ng/ml) for 6 h. The whole cell extracts (100 μg) were used to determine the amount of phospho-Syk by Western blot. The same blow was reprobed to detect the amount of Syk and tubulin (I).

daidzein, or quercetin, inhibited IKK activity (Fig. 2E, lower panel). These inhibitors, except daidzein, inhibited IL-8-induced Lck activity (Fig. 2E, upper panel). These data suggest that biochanin inhibits both IKK and Lck activities. Biochanin, but not genistein, both known inhibitors of protein tyrosine kinase [32], potently inhibited IL-8-induced IKK activity from 5 fold ($p < 0.005$) as detected by *in vitro* kinase assay using GST-IκBα as substrate, without altering the amount of IKKα in the similar treatment (Fig. 2F1 and F2). I have then tested the activity of Lck upon biochanin and genistein treatment. IL-8 induced 7 fold ($p < 0.001$) activation of Lck and both these compounds equally inhibited IL-8-induced Lck activity as determined *in vitro* Lck activity by autophosphorylation of pull-down Lck (Fig. 2G1 and G2) and Syk activity as shown by Western blot by using phospho-Syk antibody (Fig. 2I). The IL-8-induced increased amount of ICAM1 and cox2 was completely inhibited by biochanin, but not by genistein (Fig. 2H). These data further suggest that these flavonoids are potent inhibitor of PTK, but rarely inhibit Ser/Thr kinase, like biochanin.

3.4. Biochanin inhibits NF-κB activation by different inducers

To understand the effect of biochanin on NF-κB activation mediated by both Ser/Thr and PTK kinases, I have used several inducers of NF-κB that act through these kinases. Biochanin potently inhibited PMA, LPS, and H_2O_2-mediated NF-κB DNA binding (Fig. 3A) and NF-κB-dependent luciferase activity in *NF-κB-luciferase* construct transfected Jurkat cells (Fig. 3B) at 10 μM concentration, but partially inhibited TNFα-induced NF-κB activation. Even, biochanin completely inhibited pervanadate and H_2O_2-mediated NF-κB activation (Fig. 3C). These data suggest that biochanin inhibited NF-κB activation mediated by several inducers.

3.5. Biochanin, but not genistein inhibits IKK activity

As IKK activation *i.e.* phosphorylation at $Ser^{32/36}$ and Tyr^{42} are important and both Ser/Thr and PTK are involved, the effect of biochanin and genistein are determined upon stimulation with IL-8 and PV. PV is known to phosphorylate IκBα at Tyr^{42} residue [6]. I have checked the effect of biochanin and genistein upon *IKK*- and *IκBα-DN*-transfected cells. Biochanin, but not genistein, completely inhibited IL-8-induced NF-κB DNA binding and IKK activity in vector- and *IKK*-transfected cells (Fig. 4A, left and middle panels). Cells, transfected with *IκBα-DN* did not show any increase in NF-κB DNA binding, whereas, IL-8-induced IKK activity was suppressed by biochanin, but not by genistein (Fig. 4A, right panels). IL-8-induced IKK activity was completely inhibited by biochanin and partially by genistein (Fig. 4B). PV-induced IKK activity was completely inhibited by biochanin and genistein either alone or in combination (Fig. 4C). These data suggest that biochanin inhibited IKK activity completely, but by genistein only partially. Jurkat cells, transfected with *p65* and *NF-κB-luciferase* constructs were treated with different concentrations of biochanin for 6 h showed no inhibition of luciferase activity (Fig. 4D1) or NF-κB DNA binding (Fig. 4D2). The p65-overexpressed cells showed 8.8 ($p < 0.005$) fold activation of luciferase activity. These data suggest that effect of biochanin lies more upstream of p65.

3.6. Biochanin completely, but formononetin partially inhibits IKK activity

As formononetin has methoxy group, like biochanin, I have checked its effect on IKK and Lck activities. Biochanin completely, but formononetin partially inhibited IL-8-induced IKK activity as detected by *in vitro* IKK activity assay in the IL-8-induced cell extract using GST-IκBα as substrate (Fig. 4E1). Both biochanin and

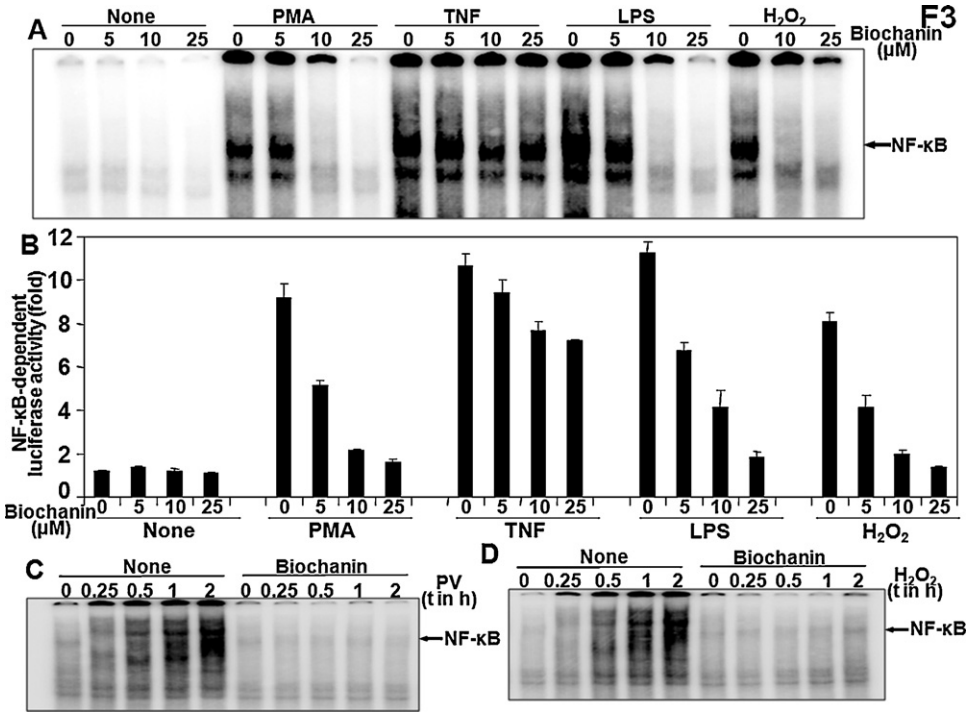

Fig. 3. Effect of biochanin on different activators (PMA, serum-activated LPS, TNFα, and H_2O_2) induced activation of NF-κB. Jurkat cells, transfected with plasmids for *NF-κB-luciferase* and *GFP*. After washing, cells were cultured for 12 h. The GFP positive cells were counted and transfection efficiency was calculated. Cells were preincubated with different concentrations of biochanin for 6 h, followed by stimulated with PMA (25 ng/ml), TNFα (100 pM), serum-activated LPS (100 ng/ml), and H_2O_2 (100 μM) for 2 h. Nuclear extracts were prepared and tested for NF-κB DNA binding (A). Luciferase activity was measured from whole cell extract (B). Jurkat cells were pretreated with biochanin (10 μM) for 6 h and then stimulated with PV (100 μM) (C) or H_2O_2 (100 μM) (D) for different times. Nuclear extracts were prepared and assayed for NF-κB DNA binding.

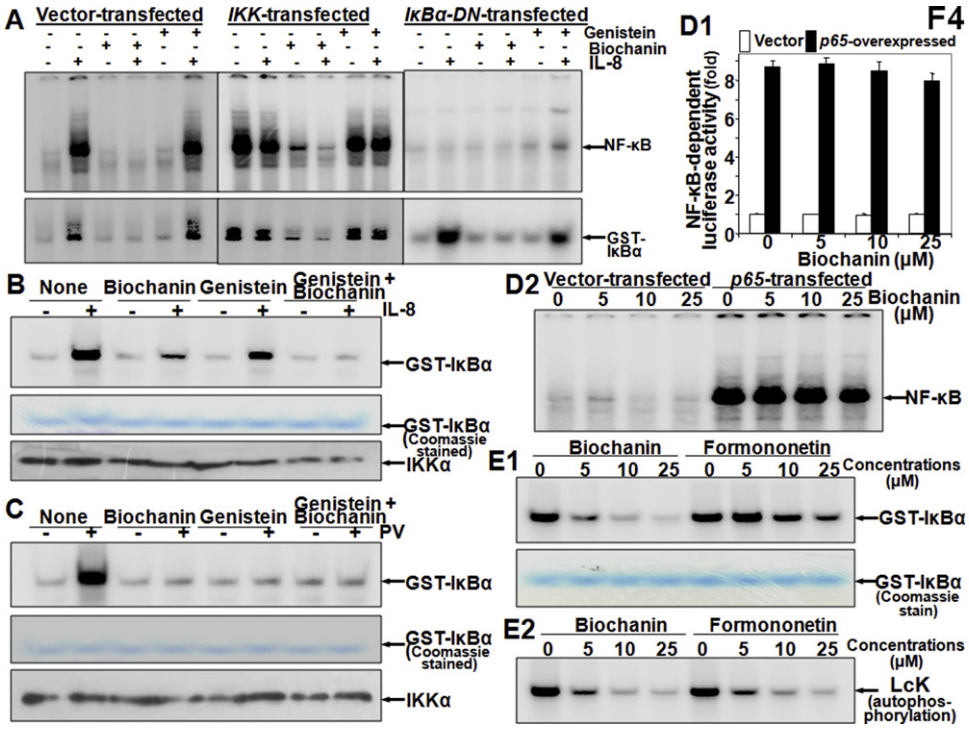

Fig. 4. Effect of biochanin and genistein on IL-8- or pervanadate (PV)-induced IKK activity. Jurkat cells, transfected with vector, *IKK*, or *IκBα-DN* constructs, were pretreated with 10 μM of biochanin or PV for 6 h and then stimulated with IL-8 (100 ng/ml) for 6 h. Nuclear extracts were assayed for NF-κB DNA binding (A, upper panels) and whole cell extracts (300 μg proteins) were used to determine the IKK activity as detected by *in vitro* IKK activity assay using GST-IκBα as substrate (A, lower panels). Jurkat cells were pretreated with 10 μM of biochanin and/or genistein for 6 h and then stimulated with IL-8 (100 ng/ml) (B) or PV (100 μM) (C) for 6 h. Whole cell extracts were used to detect the activity of IKK by *in vitro* kinase assay using GST-IκBα as substrate and the amount of IKKα was measured from 100 μg extracts from the same samples by Western blot. Jurkat cells were transfected with *vector* or *p65* construct and *NF-κB-luciferase* reporter gene construct for 3 h and then cultured for 12 h. Cells were treated with different concentrations of biochanin for 6 h. The whole cell extracts were assayed for luciferase activity and indicated as fold of activation (D1). NE were assayed for NF-κB DNA binding by gel shift assay (D2). Jurkat cells, treated with different concentrations of biochanin or formononetin for 6 h were stimulated with 100 ng/ml IL-8 for 2 h. The whole cell extracts were used to pull down IKK complex using 1 μg of each of anti-IKKα and -IKKβ antibodies or to pull down Lck by using 1 μg of anti-Lck antibody. IKK activity was assayed using GST-IκBα as substrate (E1) and autophosphorylation of Lck was detected (E2).

formononetin completely inhibited IL-8-induced Lck activity (Fig. 4E2).

3.7. Effect of biochanin and C3-toxin on IL-8 or TRAF-mediated NF-κB activation

Clostridium botulinum C3 transferase is a known inhibitor for Rho-GTPase [5]. G-protein coupled receptors usually activate cellular GTPase upon ligand interaction [33]. The role of C3-toxin on IL-8-mediated NF-κB activation and also in the biochanin-mediated inhibition of NF-κB was determined. C3-toxin partially inhibited IL-8-induced NF-κB DNA binding. Biochanin alone had no effect on the basal amount of NF-κB DNA binding. Suboptimal (5 μM) concentration of biochanin partially inhibited IL-8-induced NF-κB binding. C3-toxin, when co-incubated with suboptimal concentration of biochanin, completely inhibited IL-8-induced NF-κB DNA binding (Fig. 5A). These data suggest that IL-8-induced NF-κB activation also have role in GTPase, beside Ser/Thr kinase and PTK. Biochanin partially inhibited TRAF2-mediated activation of NF-κB, but completely inhibited TRAF6-mediated NF-κB activation (Fig. 5B). These data suggest that biochanin is a strong inhibitor of TRAF6-mediated NF-κB activation.

3.8. Genistein inhibits EGF-, but not TNFα-induced AP-1 activation

As I have seen that genistein is unable to inhibit IL-8-induced AP-1 and NF-κB activation, the effect of genistein on TNFα- and EGF-induced AP-1 activation was determined. EGF induced AP-1 DNA binding 3.8 fold ($p < 0.05$) which is possibly *via* activation of upstream receptor tyrosine kinase followed by MAPK activation [9,34]. Genistein potently inhibited EGF-, but not TNFα-induced AP-1 DNA binding (Fig. 5C). These data suggest that genistein is specific inhibitor of EGF-induced cell signaling, but not TNFα-induced.

3.9. Interaction of biochanin and genistein with proteins of Ser/Thr kinase and PTK

The AutoDock study of biochanin and genistein showed that they were docked within the Ser/Thr kinase protein. Their AutoDock energies (ΔGb, kcal/mol) and inhibition constants (*Ki*)

Table 1
Energy values for the docking interaction between serine/threonine kinase and biochanin or genistein.

Protein	Ligand	Autodock score (kcal/mol)	RMSD	Inhibition constant (*Ki*) in μM	No. of hydrogen bonds formed	Interacting residues
Ser/Thr kinase (PDB ID: 2QON)	Biochanin A	−5.48	0.02	0.082	4	Asp^{746}, Ala^{748}, Arg^{750} and $Asn7^{51}$
		−4.32	0.23	0.062	4	Asp^{746}, Ala^{748}, Arg^{750} and $Asn7^{51}$
	Genistein	−3.09	0.10	0.043	2	Arg^{750} and $Asn7^{51}$
		−2.12	0.22	0.031	1	Arg^{750}

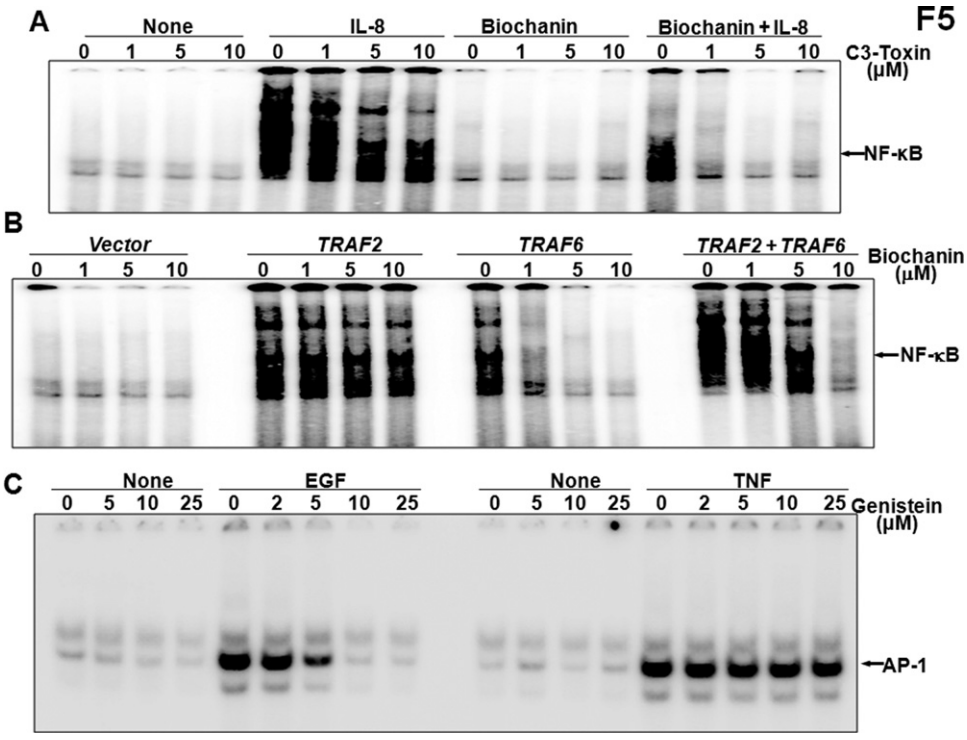

Fig. 5. Effect of C3 toxin on biochanin-mediated decrease in NF-κB DNA binding and role of TRAFs biochanin-mediated inhibition of NF-κB DNA binding. Jurkat cells, pretreated with different concentrations of C3-toxin for 3 h were treated with biochanin (5 μM) for 6 h. Cells were then induced with IL-8 (100 ng/ml) for 12 h. Nuclear extracts were used to measured NF-κB DNA binding. Jurkat cells, transfected with *TRAF2* and/or *TRAF6* constructs were treated with different concentrations of biochanin for 6 h. Nuclear extracts were prepared and assayed for NF-κB DNA binding (B). Jurkat cells were treated with different concentrations of genistein for 6 h and then stimulated with 100 pM of TNFα or 100 nM of EGF for 12 h. Nuclear extracts were prepared and AP-1 DNA binding was assayed (C).

were obtained (Table 1). Among the two ligands, biochanin exhibited the lowest free energy between −5.48 and −4.32 kcal/mol at RMSD 0.082, 0.043. In other words, biochanin possess the highest potential binding affinity into the binding site (Asp^{746}, Ala^{748}, Arg^{750} and Asn^{751}) of the Ser/Thr kinase (Fig. 6A, left panels). The higher affinity is presumably attributed to the formation of more and/or tighter hydrogen bonds between the biochanin and active site amino acids of Ser/Thr kinase. The genistein showed less binding affinity than the biochanin whereas it showed only two hydrogen bonding interaction with active site amino acid Arg^{750} and Asn^{751} (Fig. 6A, right panels). AutoDock revealed 22 energetically favorable biochanin and genistein binding models, and 12 residues were identified in all binding models among them Asp^{623}, Ala^{625}, Arg^{627} and Asn^{628} more frequently involved in formation of strong hydrogen bond with biochanin and genistein. As shown in Table 2, genistein exhibited

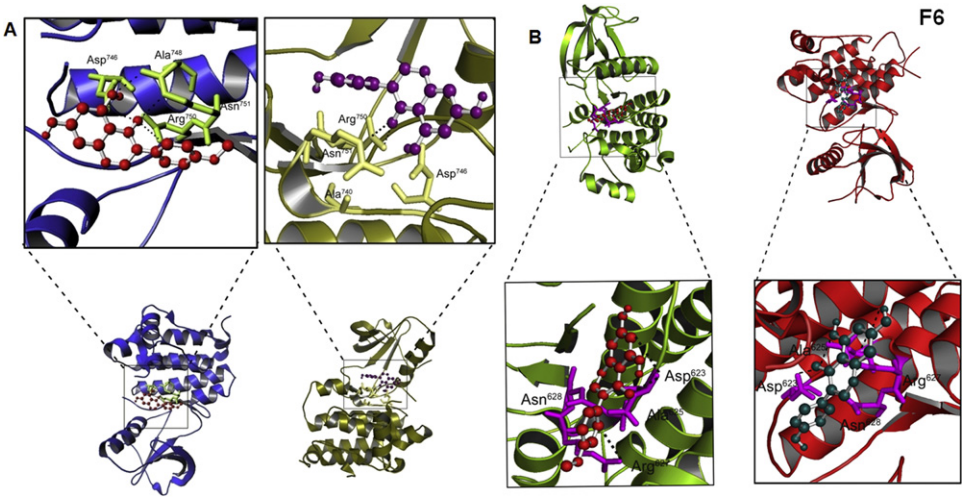

Fig. 6. Interaction of biochanin and genistein with Ser/Thr and tyrosine kinases. The view of Ser/Thr kinase (ribbon-shaped cartoon) interaction with biochanin and the zoom view reveals the active site amino acids (grey-white color sticks) forming a hydrogen bonds with biochanin (dark-grey balls with sticks) (A, left panel). The view of Ser/Thr kinase (ribbon-shaped cartoon) interaction with genistein and the zoom view reveals the hydrophobic pocket of Ser/Thr kinase (white color sticks) forming a hydrogen bond with genistein (black balls with sticks) (A, right panel). The view of tyrosine kinase (ribbon-shaped cartoon) interaction with biochanin and the zoom view reveals the active site amino acids (dark-grey color sticks) forming a hydrogen bonds with biochanin (dark-grey balls with sticks) (B, left panel). The view of tyrosine kinase (ribbon-shaped cartoon) interaction with genistein and the zoom view reveals the hydrophobic pocket of tyrosine kinase (dark-grey color sticks) forming a hydrogen bond with genistein (black balls with sticks) (B, right panel).

Table 2
Energy values for the docking interaction between tyrosine kinase and biochanin or genistein.

Protein	Ligand	Autodock total binding energy	RMSD	Inhibition constant (*Ki*) in μM	No. of hydrogen bonds formed	Interacting residues
Tyrosine kinase (PDB ID: 3GQL)	Biochanin A	−6.32	0.00	0.094	3	Asp^{623}, Arg^{627} and Asn^{628}
		−5.81	0.18	0.086	2	Asp^{623}, and Asn^{628}
	Genistein	−6.78	0.25	0.101	4	Asp^{623}, Ala^{625}, Arg^{627} and Asn^{628}
		−6.36	0.01	0.031	3	Asp^{623}, Ala^{625}, and Arg^{627}

the lowest free energy of −6.78 kcal/mol at RMSD 0.25 (Fig. 6B, left panels) by forming 4 strong hydrogen bonds with active site amino acids Asp^{623}, Ala^{625}, Arg^{627} and Asn^{628}. Biochanin also showed strong interaction with active site residues by forming the lowest free energy of −6.32 kcal/mol at RMSD 0.01(Fig. 6B, right panels).

4. Discussion

Plant derived substances, especially isoflavones, have received significant attention as prospective therapeutic agents for several types of diseases including cancers, heart diseases, diabetes, *etc.* [35,36] that are also associated with the inhibition of phosphorylation of several cellular proteins. Protein tyrosine kinases are often targeted by these isoflavones, namely genistein, erbstatin, quercetin, biochanin, *etc*. Obvious question is that if there is genistein, which is a potent inhibitor of PTK, then why plants have methoxy form of this natural organic isoflavone, *i.e.* biochanin! Plants might outsmart animals by having these molecules as toxin. But these can be utilized as potential therapeutics. Genistein and biochanin are natural forms of phytoestrogen and, as food source, are considered beneficial against several cancers, especially breast cancer.

The PTK family of kinases is mainly activated by growth factors and they induce cell proliferation. Several PTK inhibitors like genistein, erbstatin, quercetin, *etc.* suppress cell growth at different potency [11,14]. Daidzein is a structural analog of genistein that lacks PTK inhibitory activity. The data suggest the potency of the inhibition of PTK, the Lck, by these inhibitors. Biochanin, the methoxy form of genistein potently inhibited not only Lck and Syk, but also IKK, suggesting its action to inhibit both PTK and Ser/Thr kinases. Though, previous report suggests that genistein inhibits TNFα-induced IκBα degradation [32], I did not find any inhibition of IKK activity by genistein. The concentration used for this study is almost 15 times less than the reported (2.7 μg/ml *versus* 40 μg/ml). TNFα-induced cell signaling is predominantly going through activation of Ser/Thr kinase. Potential Tyr^{42} residue of IκBα is phosphorylated followed by degraded through pervanadate-mediated activation of IKK complex [6]. Higher concentration of genistein might block IKK complex, but at lower concentration only methoxy genistein, *i.e.* biochanin is able to block IKKs activity. The other methoxy phytoestrogen, formononetin, is less effective to inhibit IKK. Structurally, formononetin lacks hydroxyl group, though biochanin has two hydroxyl groups. However, the methoxy group along with hydroxyl groups might be important determinants for maximum inhibition of Ser/Thr kinase. AP-1 activation is required activation of upstream MAPK and JNK. Genistein, unlike biochanin, was unable to inhibit AP-1 activation as the upstream kinases as predominantly Ser/Thr kinases. Genistein was unable to inhibit TNFα-induced NF-κB and AP-1 activation, but potently inhibited EGF-mediated AP-1 activation as EGF requires upstream PTK activation to activate downstream Ser/Thr kinases like MAPK and JNK, whereas TNFα activates these Ser/Thr kinases by recruitment of TRAF2 [23].

In silico data suggested that compare to genistein, biochanin interacts with Ser/Thr kinase more potently, though the interactions with PTK are equal with these two isoflavones. Phosphorylation of IκBα at Tyr^{42} residue by upstream PTK possibly by Lck, Fyn, *etc.*, which is not detected actively. The PTK like Syk is known to phosphorylate Tyr^{42} of IκBα [7]. Different PTK inhibitors at lower concentration might not inhibit Ser/Thr kinase, but biochanin potently inhibited activity of IKKs by suppressing both PTK and Ser/Thr kinases. Biochanin inhibits cell signaling almost by all inducers, irrespective of PTK or Ser/Thr kinase activators. IL-8 interacts with it G-protein coupled receptors, IL-8Rs. It activates small GTPase through activation of PTK and also induces NF-κB *via* recruitment of IRAK1-TRAF6 pathway [4]. C3-toxin, a Ras-GTPase inhibitor [5], inhibits activation of NF-κB induced by IL-8 partially, which suggests that NF-κB activation by IL-8 is predominantly going through TRAF6 pathway, but not by Ras-GTPase. IL-8 activates AP-1 *via* recruitment of TRAF6, independent of PTK, which is inhibited by biochanin, but not by genistein. Biochanin potently inhibited TRAF6-induced NF-κB activation. Though, biochanin inhibited several inducers like PMA, H_2O_2, PV, *etc.* mediated NF-κB activation, it is unable to inhibit activation of NF-κB mediated by TNFα or TRAF2 potently. TNFα or TRAF6 mediated cell signaling has shown to proceed through Ser/Thr kinase. PV is known to phosphorylate IκBα at tyrosine residue [6]. PV increased activity of IKK and genistein or biochanin potently inhibited PV-induced IKK activity. How PV activates IKK and does it induce activity of Tyr kinase only or Ser/Thr kinase too, need to study further. However, several of these inducers target some common intermediate molecule, like IKK complex. Biochanin is unable to block NF-κB activation in p65-overexpressed cells which suggests that the activity of biochanin lies upstream of p65. It has no role in the NF-κB DNA binding ability *in vitro*, but suppression of NF-κB requires cellular system as it inhibits IKK complex. Biochanin potently inhibited IKK activation and inhibits IκBα phosphorylation followed by its degradation. The p65–p50 dimer is important for inducible expression of several NF-κB-dependent genes remains arrested in the resting cells with IκBα. Though biochanin inhibits translocation of p65 from cytoplasm to nucleus, the amount of p50 remains abundantly in both cytoplasm and nucleus, without interfering NF-κB(p65–p50)-mediated inducible gene expression. Biochanin did not interfere NF-κB activation in p65-overexpressed cells, further suggesting its effect lies upstream. In *IκBα-DN* transfected cells, IL-8 increases IKKs activity and biochanin completely inhibits IL-8-induced IKKs activity, but not by genistein. At any conditions, NF-κB activity is not shown because of the suppressive ability of IκBα-DN. The IκBα-DN lacks Ser32 and Ser36, so IKKs cannot phosphorylate IκBα in the transfected cells and arrest NF-κB (predominantly p50–p65) at cytoplasm. In these transfected cells, however, IL-8 induces IKK activity and that is suppressed by biochanin, but not genistein. These data further suggest that biochanin inhibits IKKs and thereby inhibits its Ser/Thr kinase activity. Previously, we have shown that several NF-κB inhibitors do not inhibit H_2O_2-mediated NF-κB activation. From this observation, it may say that H_2O_2 might be activating some PTKs, like Lck, Syk, *etc.*, those phosphorylate IκBα for its degradation and thereby NF-κB activation. The NF-κB inhibitors like silymarin [37], lapachone [38], anethole [39], and oleandrin [40] might inhibit only Ser/Thr

kinase, thereby inhibits IKK activity induced by TNFα, IL-1α, or LPS. Biochanin completely inhibited H_2O_2-mediated NF-κB activation, suggesting its role in PTK inhibition, like genistein.

Overall, the data suggest that biochanin is a novel naturally occurring isoflavone which has both PTK and Ser/Thr kinase inhibitory activities. Thus, the cytokines, like IL-8 and growth factors those are not only activating PTK, but also the Ser/Thr kinases and involve in cell signaling that leads to tumorigenic responses, might be potentially regulated by methoxy form of genistein, the biochanin. Bifunctional biochanin might be important target to regulate cellular physiology by inactivating both Ser/Thr and tyrosine kinases at very lower concentrations where no toxic effect is shown. Thus, having double-edged sword effect of biochanin to inhibit cellular kinases may be useful to regulate several biological responses those are deleterious to cells and use this molecule as therapeutic.

Acknowledgements

This work was supported by the core grant of Centre for DNA Fingerprinting and Diagnostics (CDFD). I thank Prof. T. Ramasarma, Distinguish Professor, CDFD for valuable suggestions. I thank Mr. B. Babajan, SK University, Anantapur, India for helping me in the docking study.

References

[1] Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2010;147:1117–34.
[2] Bosscher KD, Barghe WV, Haegeman G. Cross-talk between nuclear receptors and nuclear factor κB. Oncogene 2006;25:6868–86.
[3] Charalambous C, Pen LB, Su YS, Milan J, Chen TC, Hofman FM. Interleukin-8 differentially regulates migration of tumor-associated and normal human brain endothelial cells. Cancer Cell 2005;65:10347–54.
[4] Manna SK, Ramesh GT. Interleukin-8 induces nuclear transcription factor-κB through TRAF6-dependent pathway. J Biol Chem 2005;280:7010–21.
[5] Wilde C, Chhatwal GS, Schmalzing G, Aktories K, Just I. A novel C3-like ADP-ribosyltransferase from *Staphylococcus aureus* modifying RhoE and Rnd3. J Biol Chem 2001;276:9537–42.
[6] Mukhopadhyay A, Manna SK, Aggarwal BB. Pervanadate-induced nuclear factor-kappaB activation requires tyrosine phosphorylation and degradation of IkappaBalpha. Comparison with tumor necrosis factor-alpha. J Biol Chem 2000;275:8549–55.
[7] Takada Y, Mukhopadhyay A, Kundu GC, Mahabeleshwar GH, Singh S, Aggarwal BB. Hydrogen peroxide activates NF-kappa B through tyrosine phosphorylation of I kappa B alpha and serine phosphorylation of p65: evidence for the involvement of I kappa B alpha kinase and Syk protein-tyrosine kinase. J Biol Chem 2003;278:24233–41.
[8] Chung JY, Park YC, Ye H, Wu H. All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. J Cell Sci 2002;115:679–88.
[9] Martin NP, Mohney RP, Dunn S, Das M, Scappini E, O'Bryan JP. Intersectin regulates epidermal growth factor receptor endocytosis, ubiquitylation, and signaling. Mol Pharmacol 2006;70:1643–53.
[10] Middleton Jr E, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000;52:673–751.
[11] Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Ito N, et al. Genistein, a specific inhibitor of tyrosine specific protein kinases. J Biol Chem 1987;262:5592–5.
[12] Cirone M, Zompetta C, Tarasi D, Frati L, Faggioni A. Infection of human T lymphoid cells by human herpesvirus 6 is blocked by two unrelated protein tyrosine kinase inhibitors, biochanin A and herbimycin. AIDS Res Hum Retroviruses 1996;12:1629–34.
[13] Levitzki A, Gazit A. Tyrosine kinase inhibition: an approach to drug development. Science 1995;267:1782–8.
[14] Hsu CYJ, Jacoski MV, Maguire MP, Spada AP, Zilberstein A. Inhibition kinetics and selectivity of the tyrosine kinase inhibitor erbstatin and a pyridone-based analogue. Biochem Pharmacol 1992;43:2471–7.
[15] Park J, Kim SH, Cho D, Kim TS. Formononetin, a phytoestrogen, and its metabolites upregulate interleukin-4 production in activated T cells via increased AP-1 DNA binding activity. Immunology 2005;116:71–81.
[16] Mansoor TA, Ramalho RM, Luo X, Ramalhete C, Cecilla MP. Isoflavones as apoptosis inducers in human hepatoma HuH-7 cells. Phytother Res 2011. doi: 10.1002/ptr.3498.
[17] Su SJ, Yeh TM, Lei HY, Chow NH. The potential of soybean foods as a chemoprevention approach for human urinary tract cancer. Clin Cancer Res 2000;6:230–6.
[18] Perabo FGE, Von Low EC, Ellinger J, Rucker A, Muller SC, Bastian PJ. Soy isoflavone genistein in prevention and treatment of prostate cancer. Prostate Cancer Prostatic Dis 2008;11:6–12.
[19] Moon YJ, Shin BS, An G, Morris ME. Biochanin A inhibits breast cancer tumor growth in a murine xenograft model. Pharm Res 2008;25:2158–63.
[20] Lam AN, Demasi M, James MJ, Husband AJ, Walker C. Effect of red clover isoflavones on cox-2 activity in murine and human monocyte/macrophage cells. Nutr Cancer 2004;49:89–93.
[21] Kole L, Giri B, Manna SK, Pal B, Ghosh S. Biochanin-A, an isoflavon, showed anti-proliferative and anti-inflammatory activities through the inhibition of iNOS expression, p38-MAPK and ATF-2 phosphorylation and blocking NF-κB nuclear translocation. Eur J Pharmacol 2011;653:8–15.
[22] Manna SK, Gangadharan C, Edupalli D, Raviprakash N, Navneetha T, Mahali S, et al. Ras puts brake on doxorubicin-mediated cell death in p53 expressing cells. J Biol Chem 2011;286:7339–47.
[23] Manna SK, Babajan B, Raghavendra PB, Raviprakash N, Kumar CS. Inhibiting TNF receptor associated factor 2-mediated activation of nuclear factor kappaB facilitates induction of activator protein-1. J Biol Chem 2010;285:11617–27.
[24] Manna SK, Aggarwal BB. Differential requirement for $p56^{lck}$ in HIV-tat versus TNF-induced cellular responses: effects on NF-κB, activator protein-1, c-Jun N-terminal kinase, and apoptosis. J Immunol 2000;164:5156–66.
[25] Davis TL, Walker JR, Loppnau P, Butler-Cole C, Allali-Hassani A, Dhe-Paganon S. Autoregulation by the juxtamembrane region of the human ephrin receptor tyrosine kinase A3 (EphA3). Structure 2008;16:873–84.
[26] Bae JH, Lew ED, Yuzawa S, Tomé F, Lax I, Schlessinger J. The selectivity of receptor tyrosine kinase signaling is controlled by a secondary SH2 domain binding site. Cell 2009;138:514–24.
[27] Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The Protein Data Bank. Nucleic Acids Res 2000;28:235–42.
[28] Lindahl E, Hess B, van der Spoel D. Gromacs 3.0: a package for molecular simulation and trajectory analysis. J Mol Mod 2001;7:306–17.
[29] Schuettelkopf AW, van Aalten DMF. PRODRG a tool for high-throughput crystallography of protein-ligand complexes. Acta Crystallogr 2004;60:1355–63.
[30] Guest MF, Harrison RJ, van Lenthe JH, van Corler LCH. Computational chemistry on the fps-x64 scientific computers – experience on single-processor and multiprocessor systems. Theor Chim Acta 1987;71:117.
[31] Morris G, Huey R, Lindstrom W, Sanner M, Belew R, Goodsell D, et al. AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. J Comp Chem 2009;30:2785–91.
[32] Natarajan K, Manna SK, Chaturvedi MM, Aggarwal BB. Protein tyrosine kinase inhibitors block tumor necrosis factor-induced activation of nuclear factor-kappaB, degradation of Ikappa B alpha, nuclear translocation of p65, and subsequent gene expression. Arch Biochem Biophys 1998;352:59–70.
[33] Chiariello M, Vaque JP, Crespo P, Gutkind JS. Activation of Ras and Rho GTPases and MAP kinases by G-protein-coupled receptors. Methods Mol Biol 2010;661:137–50.
[34] Goh LK, Huang F, Kim W, Gygi S, Sorkin A. Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. J Cell Biol 2010;189:871–83.
[35] Corbett JA, Kwon G, Marino MH, Rodi CP, Sullivan PM, Turk J, et al. Tyrosine kinase inhibitors prevent cytokine-induced expression of iNOS and COX-2 by human islets. Am J Physiol 1996;270:C1581–87.
[36] Anderson JW, Johnstone BM, Cook-Newell ME. Meta-analysis of the effects of soy protein intake on serum lipids. N Engl J Med 1995;333:276–82.
[37] Manna SK, Mukhopadhyay A, Van NT, Aggarwal BB. Silymarin suppresses TNF-induced activation of NF-kappa B, c-Jun N-terminal kinase, and apoptosis. J Immunol 1999;163:6800–9.
[38] Manna SK, Gad YP, Mukhopadhaya A, Aggarwal BB. Suppression of tumor necrosis factor-activated nuclear transcription factor-kB, activator protein-1, c-Jun-N-terminal kinase and apoptosis by β-Lapachone. Biochem Pharmacol 1999;57:763–74.
[39] Chainy GBN, Manna SK, Chaturvedi MM, Aggarwal BB. Anethole blocks both early and late cellular responses transduced by tumor necrosis factor: effect on NF-κB, AP-1, JNK, MAPKK and apoptosis. Oncogene 2000;19:2943–50.
[40] Manna SK, Sah NK, Newman RA, Cisneros A, Aggarwal BB. Oleandrin suppresses activation of nuclear transcription factor-κB, activator protein-1, and c-Jun N-terminal kinase. Cancer Res 2000;60:3838–47.