Experimental and Computational Studies on Newly Synthesized Resveratrol Derivative: A New Method for Cancer Chemoprevention and Therapeutics?

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Abstract

Nature has been a provenance of medicinal agents for thousands of years. Resveratrol (RESL) is a naturally occurring polyphenolic compound in food stuffs such as peanuts, seeds, berries, grapes, and beverages (red wine). RESL has received significant attention due to a plethora of in vitro and in vivo reports on its cancer chemopreventive and therapeutic properties. In the present study, diacetate RESL derivative (RESL43) was synthesized. The RESL43 displayed potent cytotoxicity and triggered apoptosis in U937 cells as evidenced by poly (ADP-ribose) polymerase (PARP) cleavage, DNA fragmentation, morphological changes, and activation of FasR and FasL genes. The electrophoretic mobility shift assay revealed the suppression NFkB activity in U937 cells after treatment with RESL43 in corroboration with the deactivation of NFkB dependent genes such as IL-8, TNFR, and TNFα. Furthermore, molecular docking and dynamics studies have shown that RESL and RESL43 might exert their inhibitory activity on NFkB by altering the intramolecular binding abilities between DNA and NFkB. Taken together, RESL43 can have greater putative activity than parental RESL in a context of cancer chemoprevention and therapeutics. We suggest that the diacetate resveratrol derivative RESL43 warrants further evaluation in preclinical and clinical bridging studies in the near future.

Introduction

Nature has been a provenance of medicinal agents for thousands of years. A significant number of chemotherapeutic drugs have been isolated from natural sources. As a plant micro component, resveratrol (3,5,4’-trihydroxy-trans-stilbene) (Fig. 1), a stilbene derivative exhibits variety of biological activities (Mokni et al., 2007; Simoni et al., 2006). Resveratrol (RESL) is a polyphenolic compound produced by certain species of plants and is found particularly in polygonum roots, seeds, peanuts, berries, grapes, and subsequently present in beverages (red wine) or human diet (Latruffe et al., 2002).

RESL reportedly exhibits cancer chemopreventive effects by halting cellular events related with tumor initiation, progression, and promotion (Alkhalaf et al., 2008; Ciolino and Yeh, 2001). It has also been shown that the physiological quantities of RESL can regulate the various cellular pathways such as DNA synthesis, cell death, cell survival, cell cycle, phase I and phase II detoxification reactions, and inflammatory response by regulating nuclear fragment kappa B (NFkB) levels (Bhat and Pezzuto, 2001). Despite the tremendous advantages, the drawback with RESL is its relative lack of potency and required higher concentrations for inhibiting different target proteins (Masferrer et al., 1994; Smith et al., 1998). In addition to this, RESL has limited bioavailability and half-life in the liver. Previous reports have shown that circulating RESL have a serum half-life of 8–14 min, because its free hydroxyl groups are rapidly metabolized by sulfation and glucuronidation (Marier et al., 2002; Walle et al., 2004; Yu et al., 2002). One potential solution to these limitations is to develop RESL derivatives that can exhibit selective targeting.
with significantly greater potency and increased bioavailability (Kang et al., 2009). Moreover, compounds that are closely structurally identical to RESL may have similar or more biological effects and might demonstrate a greater pharmacokinetic profile than RESL. For instance, 3,5,4′-trimethoxy-trans-stilbene is a natural compound from the *Petrolobium hexapetalum* plant. This compound, which structurally resembles RESL, has methoxyl groups instead of hydroxyl groups at positions 3, 5, and 4 (Fulda, 2010). It is reported these compounds have greater antioxidant, antineoplastic, and antiangiogenic activities (Bader et al., 2008; Belleri et al., 2005; Cardile et al., 2007; Pettit et al., 2002) in different cancer cells. Additionally, *in vivo* studies have also shown that polymethoxystilbenes undergo different metabolic conversions and possess a higher bioavailability and half-life than RESL (Walle, 2007; Wen and Walle, 2006). Recently synthesized acetylated methoxy forms of RESL showed a promising antitumor property by inhibiting the cancer cell proliferation; the rate of inhibition of cancer cells by these derivatives is far greater than RESL (Colin et al., 2008; 2009; Marel et al., 2008). This suggests that the addition of reactive groups to the OH group of RESL can change the strength and potential activity of RESL. Hence, we have synthesized a RESL derivative with an aim to increase its chemopreventive activity compared with RESL.

**Materials and Methods**

In the present study, we used both *in silico* and *in vitro* methods (Supplementary Fig. SF1; supplementary material is available online at www.liebertpub.com/omi) to understand the potential activity of newly synthesized RESL derivatives.

**Hardware used for computational studies**

In the present *in silico* work, all computational calculations such as design and optimization of lead molecules, energy minimization, protein–ligand interaction studies (molecular docking and molecular dynamics (MD) simulations) were carried out with high frequency computational tools (System Hi-end server - 3.4 MHzs, AMD Athlon 64 bit, Quadra processor with 12 GB RAM).

**Experimental studies**

RESL (99% pure, 100 mg), K$_2$CO$_3$, acetone, propargyl bromide, EDTA, propidium iodide (PI), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), glycine, oleandrin (Oln), dimethyl sulfoxide (DMSO), streptomycin, and penicillin were obtained from Sigma. DMEM, RPMI 1640, and fetal bovine serum (FBS) were obtained from Invitrogen. Antibodies against ADF-ribose polymerase (PARP) and double-stranded NFkB were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). RESL and its derivative were dissolved in DMSO prepared after dissolving the diluted 1:1000 from stock prepared in DMSO for 24 h at 37°C.

**Organic synthesis of resveratrol derivative**

The RESL derivative (RESL43) was synthesized by heating a mixture of RESL (99% purity; 114 mg, 0.5 mmol) and K$_2$CO$_3$ in dry acetone (4.0 mL), added with acetic anhydride in reflux. After completion of initial material, the reaction mixture was evaporated, and by using column chromatography (eluting with chloroform:MeOH (10:1)) the crude product was purified to isolate ((E)-5-(4-acetoxystyril)-1,3-phenylene diacetate (20 mg) as a colorless amorphous powder. In order to study the drug-like properties of the RESL derivative, the theoretical analysis (Zmuidinavicius et al., 2003) was performed using Lipinski’s rule of five: absorption, distribution, metabolism, excretion, toxicity (ADME-Tox) (Li and Segall, 2002). The Lipinski’s Rule of five (Lipinski, 1997) demonstrates that, in general, an active drug should not have more than 5 hydrogen bond donors (OH and NH groups), a molecular weight under 500 g/Mol, not more than 10 hydrogen bond acceptors (notably N and O), and a partition coefficient log P less than 5. These parameters checked through molinspiration (www.molinspirat-ion.com) and ADME-Tox server (www.iIab.acdlabs.com/iLab2/).

**Biological evaluations**

**Cell viability assay.** The cytotoxic assay was performed on U937 cells, after initial plating as reported previously (Manna et al., 2000). Briefly, 200 μL aliquots of a suspension of exponentially growing cells were seeded in 96-well plates and incubated for 12 h. 10 μL aliquots of RESL and RESL43 at various concentrations (1 μM, 3 μM, 5 μM, and 10 μM) were treated on U937 cells for 48 h. After incubation, 200 μL MTT solution (5 mg/ml in PBS) was added to each well, and the plates were incubated at 37°C for 4.5 h. After the incubation, 100 μL DMSO was added to each well to dissolve the formazan, and absorbance was read at 550 nm using a spectrophotometric microplate reader ( Labsystems, Finland). The 50% inhibition of cell growth (IC$_{50}$) was calculated (Chan et al., 2008) and the values are represented as the mean±standard error of the mean (S.E.M.) for three autonomous experiments.

**Nuclear staining**

The 3 mL of cultured U937 cells were incubated for 48 h with a 10 μM concentration of RESL, RESL43 in 60 mL Petri dishes. After incubation, cells were permeabilized with a mixture of methanol:acetone (1:1) at 20°C for 10 min. After treating with RESL and RESL43, the extract cells were washed with PBS buffer and 0.12 μg/mL of PI was added into each well and incubated at 37°C for 30 min in the dark. The treated cells were detected by fluorescence inverted microscope at 400X magnification (Nutku et al., 2001).
**DNA fragmentation assay**

The U937 cells (1 x 10^6) were grown on the Petri dishes and treated with 10 μM concentrations of RESL and RESL43 for 48 h at 37°C. After incubation, cells were washed with 0.5 M PBS-EDTA. The treated cells were harvested by centrifugation at 14,000 rpm for 3 min at 4°C to collect the cell pellet, which was treated with 500 μL lysis buffer (50 mM Tris-HCl at pH 7.6, 10 mM EDTA at pH 8.0, and Triton X-100 0.5% w/v). The fragmented DNA was digested with 10 μL RNase (37°C for 2 h) and 20 μL proteinase K (37°C for 2.5 h). The DNA was precipitated with isopropanol and 5 M NaCl at −20°C overnight. The isolated genomic DNA fragments were analyzed by 2% agarose gel and were further identified by ethidium bromide under UV light (Park et al., 2001).

**PARP Western blot analysis**

After the treatment of U937 cells with RESL and RESL43 for 48 h at 37°C, the 10 μg samples of total cell lysates were separated by SDS-PAGE (10%) and transferred onto a poly-vinylidene fluoride membrane using a Mini-PROTEAN electrotransfer system (Bio-Rad). The blot was consequently blocked with 5% skim milk in 1% phosphate buffer saline (PBS) for 1 h and probed with their PARP antibodies overnight at 4°C. Detection was performed with an appropriate secondary antibody at 37°C for 1 h, followed by intensive PBS washing, after which the signal band was identified by chemiluminescence recognition system and autoradiography film (Lazebnik et al., 1994).

**Total RNA isolation and PCR analysis**

The U937 cells were treated with RESL and its derivative for 48h, followed by cellular harvesting and collection of cellular pellet. To the cell pellet (approximately 2 million cells) 800 μL of TRIzol was added and the cells were suspended in it by repeated pipetting. Later 200 μg/mL of glycogen was added, followed by vigorous vortexing or power homogenization. This was vortexed at high speed for 10 sec and the contents passed twice through a 26-gauge needle connected to a 1 mL syringe. The contents were transferred to a 1.5 mL microcentrifuge tube, and 160 μL of chloroform was added, followed by vortexing for about 30 sec. The tube was centrifuged at maximum speed for 5 min. The upper aqueous phase was transferred into a fresh microcentrifuge tube and 400 μL of ice-cold isopropanol was added and incubated at −20°C for 1 h. The RNA was pelleted by centrifugation at maximum speed at 37°C for 15 min. The supernatant was decanted and the pellet was washed with 200 μL of 70% ethanol, followed by centrifugation at maximum speed for 10 min. The supernatant was discarded and the pellet was dried under vacuum for about 5 min. The pellet was resolubilized in 30–50 μL RNase free deionized (DEPC-treated Milli-Q) water and aliquots were stored at −70°C.

The PCR was performed using primers for the following: interleukin-8 (IL-8)

5' -GACGCCTCTGTTGAAGGTGCA-3'; (→)
5' -CAGCACAGCTCTTCCAT-3'; (→)

tumor necrosis factor receptor (TNFR)

5' -CGCCTCAGAAAAACCACTCAGAC-3'; (→)
5' -CCAAAGAAAAATGACCAGGGGC-3'; (→)

**tumor necrosis factor (TNFs)**

5' -AGCGAGCATCCCCAAAGTT-3'; (→)
5' -GGGCACGAAGGCTCATCATT-3'; (→)

Fas ligand (FasL)

5' -CCAACCGTGAAGATT-3'; (→)
5' -GCAGTAATCTCTTCTGCATC-3'; (→)

Fas receptor (FasR)

5' -TGGCTTTGICTTCTTCTTTT-3'; (→)
5' -TCACTCATTTTTGGCTTATGG-3'; (→)

**NFκB binding assay using electrophoretic mobility shift assay (EMSA)**

The U937 cells were pretreated with 10 μM concentration of RESL and RESL43. The drug-treated and -nontreated U937 cells were stimulated with 100 pM TNF for 30 min (Rudner et al., 2010). Nuclear extracts were prepared from TNF, RESL+TNF, and RESL43+TNF treated cells, and incubated with 32P end-labeled 45-mer double-stranded NFκB oligonucleotide (4 mg protein with 16 fmol DNA) from the HIV-LTR (Santa Cruz, CA). The U937 cells were treated with RESL and its derivative (RESL and RESL43). The drug-treated and -nontreated U937 cells were stimulated with 100 pM TNF for 30 min (Rudner et al., 2010). Nuclear extracts were prepared from TNF, RESL+TNF, and RESL43+TNF treated cells, and incubated with 32P end-labeled 45-mer double-stranded NFκB oligonucleotide (4 mg protein with 16 fmol DNA) from the HIV-LTR (Santa Cruz, CA). The U937 cells were pretreated with 10 μM concentration of RESL and RESL43. The drug-treated and -nontreated U937 cells were stimulated with 100 pM TNF for 30 min (Rudner et al., 2010). Nuclear extracts were prepared from TNF, RESL+TNF, and RESL43+TNF treated cells, and incubated with 32P end-labeled 45-mer double-stranded NFκB oligonucleotide (4 mg protein with 16 fmol DNA) from the HIV-LTR (Santa Cruz, CA). The U937 cells were pretreated with 10 μM concentration of RESL and RESL43. The drug-treated and -nontreated U937 cells were stimulated with 100 pM TNF for 30 min (Rudner et al., 2010). Nuclear extracts were prepared from TNF, RESL+TNF, and RESL43+TNF treated cells, and incubated with 32P end-labeled 45-mer double-stranded NFκB oligonucleotide (4 mg protein with 16 fmol DNA) from the HIV-LTR (Santa Cruz, CA). The U937 cells were pretreated with 10 μM concentration of RESL and RESL43. The drug-treated and -nontreated U937 cells were stimulated with 100 pM TNF for 30 min (Rudner et al., 2010). Nuclear extracts were prepared from TNF, RESL+TNF, and RESL43+TNF treated cells, and incubated with 32P end-labeled 45-mer double-stranded NFκB oligonucleotide (4 mg protein with 16 fmol DNA) from the HIV-LTR (Santa Cruz, CA).

**Docking of RESL and RESL43 with NFκB**

The crystal structure of Human NFκB (p65/p50) hetero dimer (Stroud et al., 2009) was retrieved from Protein Data Bank (PDB). Prior to initiating the docking studies, all heteroatoms (non-protein molecules) were removed from the protein structure, and two chains (chain A and chain B) were used for further studies. In order to employ the protein in the AutoDock package, all polar hydrogens were added with the GROMACS program (Van Der Spoel et al., 2005). The structure obtained was optimized in 1000 steps of conjugate gradient minimization, employing the GROMOS96 forcefield. After the reliable minimal force gradient was reached, the resultant coordinates of protein structure was saved. Last, atomic solvation parameters of protein and ligand were assigned using the AutoDock (Morris et al., 2009). Docking analysis of RESL and RESL43 were performed with energy minimized NFκB. The AutoDock method was implemented as described earlier (Babajian et al., 2011). The protein grid (for each atom in ligand one grid plus a desolvation map and an electrostatic) was centered on the binding site of ligand. To allow the full-extended conformation of ligand, the grid was chosen in dimension of 70x60x60 Å, this large space allows the ligand to rotate freely. Docking was performed using Lamarckian genetic algorithm with local search parameter (Huey et al., 2007) and with AutoDock empirical free energy function. The docking runs with 2,500,000 energy evaluations for each run were performed for each molecule. The best inhibitors were screened based on cluster score (RMS tolerance equal to 0.5 Å), lowest docking energy and by inhibition constant (Ki).
ligand bound form of NFκB (docked initial co-ordinates of NFκB-RESL and NFκB-RESL43 were used as ligand bound form of NFκB). The protonation states of all ionizable residues of protein were set to their normal states at pH 7. All MD simulations were performed with default gromacs forcefield (GROMOS96) using GROMACS 4 package (Van Der Spoel et al., 2005). Throughout the MD simulations, all the protein atoms were enclosed by a cubic water box of water molecules (SPC3) that extended 9.8Å from the protein, and periodic boundary environments were applied in all directions. The systems were neutralized with Cl⁻ and Na⁺ counter ions replacing the water molecules. The energy minimization was performed using the steepest descent algorithm for 10,000 steps. Furthermore, 100 ps position restrained MD run was carried out to allow the movement of solvent molecules and counter-ions by fixing the protein backbone. Finally, the equilibrated systems were subjected to 10 ns production runs with a time step of 2 fs at pressure (1 atm) and stable temperature (300°K). All simulations were run under periodic boundary conditions with V-rescale thermostat (Bussi et al., 2007) and NPT ensemble. The Van der Walls (vdw’s) forces were treated by using long-range electrostatic interactions (Essman et al., 1995) with setting of the Particle Mesh Ewald (PME) method (cutoff of 14 Å). All the dynamic analyses of the trajectories were done using the xmgrace analysis package.

**Calculation of the binding free energy**

The interaction free energies of NFκB-RESL and NFκB-RESL43 were evaluated with the linear interaction energy (LIE) method (Aqvist et al., 1994). The LIE method provides approximate estimation to the hydration and absolute binding free energies with less computational attempt. To perform the binding energy estimation for NFκB-RESL and NFκB-RESL43 complexes, we used this approach. LIE method evaluates the vdw’s and electrostatic interaction energies of the RESL and RESL43 (ligands) in free and bound states. For this purpose, we carried out MD simulations of ligands in water to measure the electrostatic and vdw’s contributions of the free ligands with equal protocol and considerations to the complex dynamics.

The approximated binding energy is obtained as it is shown below:

\[
\Delta G = \sigma(E_{vdw\text{-protein bound ligand}} - E_{vdw\text{-ligand bound sol}}) + \beta(E_{eqq\text{-protein bound ligand}} - E_{eqq\text{-ligand bound sol}})
\]

where \(<EVDW>\) complex and \(<EVDW>\) free denote the average vdw’s interaction energies in the bound and free forms, and \(<EQQ>\) complex and \(<EQQ>\) free denote the average electrostatic interaction energies in the free and bound forms. The value of \(\sigma\) strongly depends on the force field and the computational methods applied. For this, a proper value should be determined by comparing the experimental and calculated binding energies. In addition, \(\beta\) value was originally fixed to 0.5. However, the study of solvation energies of various small molecules showed that \(\beta\) value decreases with the number polar groups (hydroxy).

**Hex docking analysis on NFκB-RESL,**

**NFκB-RESL43 with DNA**

For Hex docking studies, we used coordinates of apo-form NFκB and docked initial coordinates (NFκB-RESL and NFκB-RESL43) from AutoDock. In docking analysis apo-form of an NFκB, NFκB-RESL, NFκB-RESL43 was used as a receptor and DNA (coordinates retrieved from the crystal structure 3GUT) molecule was used as ligand in these studies. The free energies were calculated based on shape complementarities only as types of association using a default grid spacing of 0.6Å. By sampling receptor and ligand about to their centroids, 180°A of total possible rotations were incremented. The steric scan \((N=20)\) phase of the docking calculation was performed at \((1+0)/0.75=53\) intermolecular separations, in positive/negative steps of 0.75Å. The final search \((N=25)\) phase was applied to the maximum scoring scan orientations in steps of 0.75°/2°. The rotational search used angular increments and twist angle of about 7.5, 5.5 degrees in ligand and receptor rotational angles. Finally, for viewing 10,000 sorted lowest docking energies, the top 2000 orientations were confined in 500 clusters. The results provided by the Hex docking server are arbitrary energy values that serve as the score and determine which among the given solution is the best fit. The best solution is judged as to which energy value possesses the greatest magnitude in the negative order (Macindoe et al., 2007).

**Results**

**Design and synthesis of RESL43**

The RESL derivative was designed and synthesized from alkylation of acetic anhydride on 3,5,4'-OH groups of RESL and the reaction product is \((E)-5-(4-acetoxystyryl)-1,3-phe-\)

nylene diacetate (RVS43) (Fig. 2).

1H NMR (400 MHz, CDCl3)

\(\delta\) ppm 2.29 (s, 9H), 6.83 (s, 1H), 6.95 (d, J = 16.24 Hz, 1H), 7.08 (m, 5H), 7.47 (d, J = 8.07 Hz, 2H); (Fig. 3) Mass: \([m/z]=371.9\) [M+H]⁺ (Supplementary Fig. SF2). The synthesized compounds were analyzed for their drug-likeness based on their physicochemical properties by using molinspiration and ADME-tox server. It was found that synthesized RESL43 strictly follow drug-likeness properties (Table 1, Table 2).

**In vitro studies**

**Cytotoxic MTT assay.** In the present study, the antitumor effects of RESL43 were investigated in U937 cancer cell lines by means of cytotoxic MTT assays. The U937 cells were treated with different concentrations of RESL and RESL43 (1 μM to 10 μM) and showed a dose-dependent cytotoxicity. Figure 4 represents the cell viability as % viability versus increasing concentrations of the drugs. RESL and RESL43 inhibited the proliferation of U937 with IC₅₀ of 32±0.78 μM and 6±0.92 μM, respectively.

**Morphological changes and DNA fragmentation in U937.** In this study, we observed that RESL, RESL43, and positive control Oli induced the morphological changes of U937 cells in shape by membrane blebbing, formation followed by apoptotic bodies (Fig. 5b, d, arrow No. 3, 4), and nuclear shrinking (Fig. 5c arrow, No. 2,3). We next examined the damage of DNA in the U937 cells after treatment with RESL and RESL43 by performing gel electrophoresis of chromosomal DNA. A clear pattern of laddering was observed only in the treated samples (RESL and RESL43), at 10 μM concentration, but none in the untreated cells (Fig. 6).

**Immunoblot analysis.** To clarify the death signaling pathway underlying the RESL43 induced apoptosis, we
observed the cleavage of PARP. The Western blotting results show the RESL43 cleaves the PARP into two fragments, determined by the diminution of its parent band (116 kDa) and the associative accumulation of its cleavage fragment (85kDa) (Fig. 7a).

Semiquantitative RT-PCR on RESL derivative decrease the expression of TNFα and IL-8 and upregulation of Pro-apoptotic genes FasR and FasL. The most potent NFkB activator is the proinflammatory cytokine TNFα and trimeric cytokine rector TNFR, which cause rapid phosphorylation at N-terminal regulatory domain of kBs site. We examined the levels of TNFα and TNFR in U937 cells after treatment with RESL43 (10 lM concentration) by using a semiquantitative RT-PCR-based assay. In this assay, PCR products are generated during both log-phase and plateau reactions by conducting different cycles (20, 25, and 35-cycle) rounds of PCR. In this result, RESL and RESL43 have been shown to reflect levels of mRNA in U937 cells. In Figure 7b, c, TNFα and TNFR bands intensities were unchanged in untreated cells, and in treated cells after 20-cycle rounds of PCR the low constitutive level of TNFα and TNFR transcripted. These data show decreased TNFα and TNFR production after treatment with RESL43 (Fig. 7b, c). Apoptotic cell death was induced by the engagement of FasR, FasL, and these apoptosis molecules play a vital role in the modulation of immune function, particularly in activation-induced cell death. Figure 7d, e shows the clear increased level of Fas and FasL genes in RESL43 treated cells, whereas the level decreased in RESL-treated cells.

IL-8 is a pleiotropic chemokine that plays a vital role in angiogenesis, as well as in the promotion of malignant cell proliferation. Our studies on IL-8 show the decrease in level of expression in U937 cells after treatment with RESL43 with very low intensity of the band (Fig. 7f).

NFkB DNA binding assay. The U937 cells were pre-treated for 48 h with different concentrations of RESL and RESL43, then stimulated with 100 pM TNF for 30 min. The nuclear extracts were prepared and assayed for NFkB by EMSA. The results show that in untreated cells, TNF induces 10-fold activation of NFkB, and in treated cells with RESL43 suppress the activity of NFkB in a dose-dependent manner (Fig. 8).

In silico studies
Secondary structure and active site analysis of NFkB (RelA(p65)/ p50). The crystal structure of a higher-order

![FIG. 2. A proposed chemical reaction in synthesis of RESLA3 from RESL.](image)

![FIG. 3. Assigned NMR Spectra of RESL43 (3-(acetyloxy)-5-{((E)-2-[4-(acetyloxy)phenyl]-ethenyl]phenyl acetate) at 400MHz ¹H spectrum.](image)
complex of NFkB bound to the HIV-1 LTR (PDB ID:3GUT) was retrieved from PDB and secondary structure elements and active site analysis were carried out using the PDB sum server (Laskowski et al., 2005). Figure 9 shows p65 monomer (Chain A) consisting of 273 amino acid with 91 strands, 9 alpha helix, 2 helix, and 171 other structural elements. The 312 amino acids p50 monomer (chain B) consists of 109 strands, 33 alpha helix, 8 helices, and 162 other structural elements. The important residues that contact with the DNA in both chains of NFkB are Met33(A), Arg34(A), Arg35(A), Tyr36(A), Cys38(A), Glu39(A), Arg41(A), Ser42(A), Ala43(A), Gly44(A), Ser45(A), Lys122(A), Lys123(A), Asn155(A), Arg187(A), Gln220(A), Glu360(B), His364(B), Gly365(B), Val442(B), Thr443(B), Lys444(B), Lys541(B), Pro543(B), Lys572(B), Gln574(B), Lys575(B), Arg605(B), and Gln606(B) (Fig. 10). The DNA wraps around two chains of NFkB, and the complex appears as a butterfly (Fig. 9). The N-terminal, nine-stranded β-barrel of the crystal structure contains the recognition loop, which binds three concomitant G:Cbp in the major groove. The electrostatic surface of NFkB was analyzed with APBS (Baker et al., 2001) by assigning amber force field (default 99 charges and 0.46 raddi). The NFkB electrostatic surface image were generated and visualized with Pymol. The major groove. The electrostatic surface of NFkB was analyzed with APBS (Baker et al., 2001) by assigning amber force field (default 99 charges and 0.46 raddi). The NFkB electrostatic surface image were generated and visualized with Pymol. The NFkB active site residues are present in distinct areas on the electrostatic potential surface of NFkB, indicating that most of the active site amino acids belong to a negative potential surface area where the DNA interactions are carried out (Fig. 10).

Docking Analysis of RESL and RESL43 with NFkB. The experimental results showed that RESL43 had better activity towards NFkB compared with RESL. However, the molecular interactions studies of RESL with NFkB are not clear. To understand the possible binding mode of RESL and RESL43 on NFkB, we performed a molecular docking analysis. The 100 docking conformations for RESL and RESL43 were separated into groups according to a 1.0 Å Root Mean Square Deviation (RMSD) principle by using the clustering module in AutoDock. Besides RMSD clustering, AutoDock also uses protein–ligand free energy estimations to identify the top binding mode. The energy parameters calculated by AutoDock4 include intermolecular energy (desolvation energy, vdw’s energy, electrostatic energy, and hydrogen bonding energy), torsion energy, and internal energy. The first and the third parameters composed of binding energy; the first two parameters build up docking energy. During these, the hydrogen bonding and hydrophobic interaction between ligand and protein is the most important, because in most cases, it can decide the binding strength and inhibition specialty to a great extent. The AutoDock energy information is listed in Table 3, and the interaction modes of RESL and RESL43 are shown in Figures 11 and 12. The compound RESL majorly interact with p50 monomer of NFkB, the 4’-OH atom of RESL, interact with the ε-amino group of Lys572(B) by forming a 2.5 Å distance H-bond and γ-amino group of Gln574(B) by forming a 3.2 Å distance H-bond. The 3-OH atom of RESL43 interacts with p50 monomer and NH1 and NH2 amino groups of Arg605(B) by forming 3.8 Å distance H-bonds. Overall, the docking modes model for RESL shows the phenolic oxygen moieties stacked to Lys572(B), Gln574(B), and Arg605(B) of the NFkBp50 monomer (Fig. 11). The ranking of binding energies for RESL with NFkB is −6.12 Kcal/Mol at 32 μM of Ki. The RESL43 had more active groups in its structure; it forms six hydrogen bonds with both chains of NFkB, the 3-(acetyloxy) group of RESL43 interacts with NH1 amino group of Arg53(A), Arg54(A), and Arg584(B), by forming <2.15 Å distance H-bonds. 2-[4-(acetyloxy)phenyl] group of RESL43 interacts with ε-amino group of Lys572(B) and γ-amino group of Gln574(B) by forming a <1.25 Å distance H-bond. Final docking energy of RESL43 with NFkB shows the maximum binding energy of −10.42 Kcal/Mol and Ki of 21.879 nM (Fig. 12).

**MD analysis.** The dynamic studies were carried out on apo-forms of NFkB, NFkB-RESL, and NFkB-RESL43 (NFkB-ligand) complexes. The stability of the system (protein, ions, water, and ligand) properties was inspected by means of RMSD, potential energy, RMS Fluctuations (RMSF), and protein is the most important, because in most cases, it can decide the binding strength and inhibition specialty to a great extent. The AutoDock energy information is listed in Table 3, and the interaction modes of RESL and RESL43 are shown in Figures 11 and 12. The compound RESL majorly interact with p50 monomer of NFkB, the 4’-OH atom of RESL, interact with the ε-amino group of Lys572(B) by forming a 2.5 Å distance H-bond and γ-amino group of Gln574(B) by forming a 3.2 Å distance H-bond. The 3-OH atom of RESL43 interacts with p50 monomer and NH1 and NH2 amino groups of Arg605(B) by forming 3.8 Å distance H-bonds. Overall, the docking modes model for RESL shows the phenolic oxygen moieties stacked to Lys572(B), Gln574(B), and Arg605(B) of the NFkBp50 monomer (Fig. 11). The ranking of binding energies for RESL with NFkB is −6.12 Kcal/Mol at 32 μM of Ki. The RESL43 had more active groups in its structure; it forms six hydrogen bonds with both chains of NFkB, the 3-(acetyloxy) group of RESL43 interacts with NH1 amino group of Arg53(A), Arg54(A), and Arg584(B), by forming <2.15 Å distance H-bonds. 2-[4-(acetyloxy)phenyl] group of RESL43 interacts with ε-amino group of Lys572(B) and γ-amino group of Gln574(B) by forming a <1.25 Å distance H-bond. Final docking energy of RESL43 with NFkB shows the maximum binding energy of −10.42 Kcal/Mol and Ki of 21.879 nM (Fig. 12).

**Table 1. Physicochemical Properties of Best RESL and RESL43**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Compound</th>
<th>LogP</th>
<th>TPSA</th>
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<th>M.W.</th>
<th>nON</th>
<th>nOHNH</th>
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<tr>
<td>2.</td>
<td>RESL43</td>
<td>4.432</td>
<td>88.76</td>
<td>35</td>
<td>490.8</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>8</td>
<td>414.5</td>
</tr>
</tbody>
</table>

| *Partion coefficient; bTopological surface area; cNumber of atoms; dMolecular weight; eH bond acceptors; fH bond donor; gNumber of rotatable bonds. |
gradually decreases from −281467.9 to −281959.9 Kcal/Mol; it indicates NFkB is energetically stable during MD simulation. The RMSD values of Cα atoms in apo-form NFkB and NFkB-RESL and NFkB-RESL43 were plotted from ∼0 to ∼10000 ps. As shown in Figure 13a, it signifies that RMSD of both systems (apo-form NFkB and NFkB-Ligand) reach equilibration at ∼4500–8000 ps. The RMSD of apo-form NFkB were calculated from ∼7700–10000 ps trajectory, where the average datum points fluctuated for NFkB at 0.96 to 0.95 nm. NFkB-RESL was calculated from ∼7100–10000 ps trajectory, average datum points fluctuated around 0.74 to 0.75 nm. Whereas the NFkB–RESL43 complex was calculated from ∼4500–10000ps.

FIG. 4. The % of death in U937 cells after treatment with different concentrations of RESL2 and RESL43.

FIG. 5. Morphological alterations of U937 cells following expose to RESL (10 μg/mL), Oln (1 μg/mL), and RESL43 (10 μg/mL) for 48 h. (a) Control U937 cells were observed by phase contrast inverted microscope. (b)–(d) The RESL, Oln, and RESL43 treated U937 cells were stained by PI. 1►: normal cells; 2►: membrane blebbing; 3►: apoptotic body; 4►: nuclear shrinking.
trajectory, average datum points fluctuated around 0.47 nm. This result indicates that the binding of RESL43 with NFkB creates the more stable conformation when compared to apo-form NFkB (Fig.13a). The Rg or compactness of structure around a group of atoms is known to decrease as stability of a folded protein increases up to a certain value. The Rg values of both systems (apo-form NFkB and NFkB–ligand) stabilized about ~3500 ps, indicating that the MD simulation accomplished equilibrium after ~3500 ps. Initially, the Rg values of apo-form NFkB and NFkB-ligand were stabilized from 2.5 to

FIG. 6. Fragmentation of genomic DNA, after treatment of U937 cell with 10 μM concentration of RESL and RESL43. M, marker; UN, untreated.

FIG. 7. (a) Effect of RESL and RESL43 on cleavage of PARP 116 kDa fragment to 85 kDa fragment. (b)–(f) TNFα, TNFR, Fas, FasR, and IL-8 expression levels after treatment with RESL and RESL43. M, marker; OLN, oleandrin (positive control); UN, untreated.
FIG. 8. The U937 cells were pretreated with TNF and RESL, RESL43, and incubated for 48 h; the nuclear extract were prepared; and NFkB DNA–protein binding activity was determined by EMSA. FP, free probe; UN, untreated.

FIG. 9. 3-D view of the NFkB p65 (yellow)/p50 (red) in complex with DNA (blue).
2.3 nm. The above results suggest that the Rg value decreased upon RESL and RESL43 interaction with NFkB. This result indicates RESL43 binding changes the microenvironment of NFkB, leading to the conformational changes in the NFkB. The time-dependent hydrogen bonding analysis on NFkB-RESL and NFkB-RESL43 shows that the complexes maintained stable binding conformation throughout the simulation, and these complexes are also able to attain contacts in a polar environment.

The RMSF of the backbone atom was plotted for apo-form NFkB and NFkB-Ligand in order to understand the dynamic stability of the amino acid residues. It was observed that, throughout the dynamic simulations, very few fluctuations exceeded 0.7 nm (Fig. 13b). The graph shows that the N-terminus residues in both A and B chains (245–260 (A) and 570–585 (B)) are flexible and have fluctuations close to 0.8 nm, while DNA binding residues showed less fluctuations in both A and B chains of NFkB after the inclusion of RESL and RESL43, and they remain stable throughout the ~10000 ps time (0.35 nm) (Fig. 14).

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Free energy calculation using LIE. The LIE is a semi-empirical MD approach for evaluation of binding free energies in between the protein–ligand complex. This method applies differences between the average electrostatic and vdw’s interaction energies between the inhibitors and their respective environments during MD simulations. This method predicts that the binding free energy can be retrieved from simulations of bound and free states of the ligand. We have used RESL and RESL43 to study interactions with NFkB. Table 4 concludes the average interaction energies between the RESL and RESL43 with their surroundings by applying the LIE formula $\Delta G$ of NFkB-RESL: $-21.1804 \text{ KJ/Mol}$, and NFkB-RESL43: $-40.0065 \text{ KJ/Mol}$.

DNA docking with a docked complex of NFkB–RESL43. To understand the molecular interactions of NFkB with DNA in the presence of RESL and RESL43, three different modes (NFkB–DNA, NFkB–RESL, and NFkB–RESL43) of Hex docking studies were performed. Energy minimized structure of NFkB first docked with the DNA molecule. The Hex results show that DNA tightly interacts with both chains of NFkB and it release the maximum total binding energy of $-3005.72 \text{ Kcal/Mol}$ (Table 5). The docked complex (NFkB–DNA) was superposed in experimental structure (PDB ID: 3GUT) and it shows RMS deviation 0.0 nm. The docked complex of NFkB—RESL redocked with DNA. Due to binding of RESL at the DNA binding site of NFkB, the conformation of NFkB does not allow the DNA to fit as tightly as in apo-form (Fig. 15). As similar to RESL results, the docked complex NFkB–RESL43 also does not allow the DNA to fit into the NFkB site and releases a binding energy of $-24.50 \text{ Kcal/Mol}$ (Fig. 16).

Table 3: Docking Energies of RESL and RESL43 with NFkB

<table>
<thead>
<tr>
<th>No.</th>
<th>Protein</th>
<th>Compound</th>
<th>Cluster$^a$</th>
<th>RMSD$^b$ (Kcal/Mol)</th>
<th>Inhibition constant$^c$ (Ki)</th>
<th>No of H bonds (drug-enzyme)</th>
<th>Amino acid involved in interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NFkB (PDB ID: 3GUT)</td>
<td>RESL</td>
<td>32</td>
<td>1.256</td>
<td>$-6.12$</td>
<td>32 $\mu$M</td>
<td>4 Lys572(B), Gln574(B) &amp; Arg605(B)</td>
</tr>
<tr>
<td>2.</td>
<td>RESL43</td>
<td></td>
<td>44</td>
<td>0.225</td>
<td>$-10.42$</td>
<td>21.879 $\text{nM}$</td>
<td>6 Arg33(A), Arg35(A), Arg354(B), Lys572(B) &amp; Gln600(B)</td>
</tr>
</tbody>
</table>

$^a$Indicative of the total number of binding modes produced; $^b$Heavy atoms root-mean-square deviation with respect to the experimental structure; $^c$The change in binding free energy is related to the inhibition constant using the equation: $\Delta G = RT \ln K_i$, where R is the gas constant 1.987 cal K$^{-1}$ Mol$^{-1}$, and T is the absolute temperature assumed to be 298.15 K; $^d$Estimated inhibition constant at 298.15 K.
Discussion

Many anticancer remedies presently in use are inadequate, not only in terms of their therapy efficacy, but also because they have undesirable side effects (Surh, 2003). However, certain dietary constituents known as phytochemicals exhibit chemopreventive and growth restrictive properties against different types of diseases, including cancer (Carmeliet, 2003; Wahl et al., 2011). Resveratrol is one of the important dietary phytochemical that is less toxic and plays a vital role in downregulation of critical genes that are activated in cancer. Further, it was also reported that due to the presence of polyphenolic groups in its structure, it shows higher levels of cytotoxic effects in different cancer cells (Kraft et al., 2009). However, it has some limitations, including its slow reactivity, short half-life, and low level of bioavailability claimed in vivo (Marier et al., 2002). One of the possible outcomes of these limitations is to develop RESL derivatives, which have more potential activity than RESL (Kang et al., 2009). Taking this into consideration, we synthesized a RESL derivative (RESL43) by substituting diacetate groups in the seed structure of RESL. The RESL43 showed higher cytotoxic effect on U937 cells when compared to parental RESL. Furthermore, RESL43 showed a lower IC₅₀ (6 μM) on U937 cells, which is five-fold better than RESL IC₅₀ value (32 μM). Our results are in corroboration with the previous report of Manna et al. (2000), where the IC₅₀ value of RESL in U937 cell line is 32 μM. Further studies have also indicated that addition of reactive groups such as methoxy and triacetate into RESL enhances cytotoxic effects in several cancer cell lines (Billack et al., 2008; Colin et al., 2009; Mazué et al., 2010). In addition to growth inhibition, we observed RESL and RESL43 induced morphological changes in U937 cells, which is a characteristic of apoptosis. It was similarly observed in a previous report that RESL induced morphological changes in different type of cancer cells by cell shrinkage, chromatin condensation at nuclear periphery, nuclear fragmentation in several membrane-bound vesicles, and formation of actin-dependent cytoplasmic protrusions, which finally separate from the cell and forms apoptotic bodies (Bhardwaj et al., 2010). In this study, RESL43 showed a clear pattern of laddering in DNA fragmentation assay. This feature is commonly associated with the apoptotic process, in which the DNA is fragmented into 180 nucleosomal units by the endogenous endonucleases.

FIG. 11. RESL binding interaction with DNA binding residues (red color sticks) of p50 chain (Chain B) of NFkB.

FIG. 12. Binding mode of RESL43 with DNA binding region of NFkB. Yellow, p65 (Chain A); Red, p50 (Chain B).
Another marker assay (PARP assay) of apoptosis confirms the induction of apoptosis in U937 cells after treatment with RESL43; it showed the cleavage of PARP into two fragments, which is an indication of activation of apoptotic enzyme caspases (Nicolini et al., 2001). Further, apoptotic regulating molecules FasR and FasL were also expressed in U937 cells after its treatment with RESL43. These results showed that RESL43 had greater cytotoxic and anti-apoptotic properties on U937 cells compared with natural RESL.

It is well known that RESL has antioxidant and anti-inflammatory effects in various cancers (De and Villegas, 2005). NFkB is a transcription factor involved in various signaling cascades and plays a crucial role in acute and chronic inflammatory processes (Aggarwal, 2004). In our studies on NFkB activity, the EMSA results showed that RESL43 the significantly suppress the level of NFkB in the presence of TNF. This might be due to interference of RESL43 in binding of NFkB with DNA. Previous studies have shown that RESL inhibit nuclear translocation of NFkB and prevent NFkB...
binding with the DNA (Holmes-Mcnary and Baldwin, 2000; Manna et al., 2000). Our studies on proinflammatory cytokine TNFz and TNFz, which play a role in the activation of NFkB, showed decreased expression levels. Further, decreased levels of IL-8 gene expression were also observed in U937-treated cells. As previous reports stated, the suppression of NFkB activation by RESL may underlie the observed decrease in TNFz, TNFz, and IL-8 levels in different cancer cells (Holtmann et al., 1999; Zhu et al., 2011). Overall, our in vitro studies demonstrate increased chemopreventive activity of RESL43 on U937 cells compared with RESL. This may be due to substitution of reactive diacetate groups on RESL, which increases biochemical, anti-tumor, pro-apoptotic, and anti-inflammatory effects in U937 cells (Roberti et al., 2003).

To understand the molecular interaction in between the NFkB, RESL, RESL43, and DNA, we performed molecular docking and MD simulation analysis. The docking analysis showed that RESL43 can enter the DNA-binding region of NFkB. In the arrangements with the lowest docked energy, RESL43 were sandwiched between the DNA binding residues Arg33(A), Arg35(A), Gln220(A), Arg246(A), Arg354(B), Lys541(B), Lys572(B), Gln574(B), Arg605(B), and Gln606(B). Overall from docking studies, we found that the binding affinity of RESL43 to NFkB is stronger than RESL to NFkB. The molecular docking analysis was in fairly good agreement with experimental findings (Piccaglia et al., 2008). The MD simulation studies showed that the apo-form of NFkB and docked complexes (NFkB–RESL, NFkB–RESL43) are stable at 10 ns. The RMSD, potential and Rg analysis showed that the complexes (NFkB–RESL and NFkB–RESL43) are stable at 10 ns. The RMSD analysis also showed flexibility of DNA binding residues reduced upon the binding of NFkB with RESL43. These results showed a correlation between increased inhibition potency of the RESL43 and its ability to stabilize the NFkB fluctuations. The LIE calculates the estimated free energy of RESL and RESL43 with NFkB in bound and free states. It was observed that during a 10 ns period, vdw’s interactions are more favorable in the NFkB-bound form than in solvent. This is predicted since in NFkB the number of interacting nonpolar groups is greater. On the other hand, we identified a decrease in electrostatic interaction energy in going from bound to the free state by RESL43 and RESL. This indicates that for RESL and RESL43 contacting several oxygen groups; the aqueous environment is favored electrostatically. Similar decrease in the electrostatic interaction energy upon binding of P450-cam and thrombi to proteins has been reported by Shi et al. (2012). Our results are well poised between the unfavorable electrostatic and favorable nonpolar interaction energies; it determines the binding affinities of these ligands to NFkB (Froloff et al., 1997). We see that the energy of these complexes remain quite stable and its value keeps fluctuating around -21 KJ/Mol to -41 KJ/Mol throughout the simulations, indicating the initial stable hydrogen bonds between ligand and protein are managed. On the whole, dynamic studies showed that RESL43 can steadily anchor NFkB in order to apply an inhibition effect of binding to DNA. The Hex docking analysis on DNA with the docked complexes of NFkB–RESL, NFkB–RESL43 showed that ability of DNA binding with NFkB is decreased in both complexes (NFkB–RESL and NFkB–RESL43). This might be due to RESL and RESL43 interfering in between NFkB and

### Table 4. Linear Interaction Energies of RESL and RESL43 with NFkB Heterodimer

<table>
<thead>
<tr>
<th>Drug</th>
<th>Evdw.bound (Kcal/Mol)</th>
<th>EElec.bound (Kcal/Mol)</th>
<th>E-total (Kcal/Mol)</th>
<th>Absolute binding energy (Kcal/Mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RESL</td>
<td>-24.54</td>
<td>-9.55</td>
<td>-9.73</td>
<td>-9.73</td>
</tr>
</tbody>
</table>

### Table 5. Hex Docking Results of NFkB-RESL and NFkB-RESL43 with DNA (*Major DNA Binding Residues)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Molecules</th>
<th>RMS</th>
<th>E-total (Kcal/Mol)</th>
<th>Amino acid in interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NFkB-DNA</td>
<td>0.028720</td>
<td>-3005.72</td>
<td>Met32(A), Arg35(A)<em>, Arg35(A)</em>, Tyr36(A), Cys38(A), Glu39(A), Arg41(A), Ser42(A), Ala43(A), Gly44(A), Ser45(A), Lys122(A), Lys123(A), Asn155(A), Arg187(A), Gln220(A), Arg246(A), Arg246(A)<em>, Arg252(A), Arg254(A), His364(A)</em>, Gly365(A), Val442(A), Thr443(A), Lys444(A), Lys454(A), Pro541(A), Lys572(A), Gly574(A), Lys577(A), Arg579(A), Arg605(A), and Gln606(B).</td>
</tr>
<tr>
<td>2</td>
<td>NFkB-RESL Complex with DNA</td>
<td>0.859802</td>
<td>-628.25</td>
<td>Met32(A), Arg35(A)<em>, Arg35(A)</em>, Tyr36(A), Cys38(A), Glu39(A), Arg41(A), Lys122(A), Asn155(A), Arg187(A), Gln220(A), Arg246(A), Arg254(A)<em>, Arg256(A), His364(A)</em>, Gly365(A), Val442(A), Thr443(A), Lys444(B), Lys445(B), Pro541(B), Lys572(B), Gly574(B), Lys577(B), Arg579(B), and Gln606(B).</td>
</tr>
<tr>
<td>3</td>
<td>NFkB-RESL43 complex with DNA</td>
<td>0.47845</td>
<td>-102.58</td>
<td>Met32(A), Tyr36(A), Cys38(A), Glu39(A), Arg41(A), Ser42(A), Ala43(A), Lys122(A), Asn155(A), Arg187(A), Gln220(A), Arg246(A), His364(A)*, Thr443(B), Pro541(B), Gly574(B), Lys577(B), and Arg605(B).</td>
</tr>
</tbody>
</table>
FIG. 15. DNA interaction with NFkB–RESL complex. Zoom bottom view represents the interaction of RESL at DNA binding region of NFkB. Zoom top view shows the binding interaction of DNA with the complex of NFkB–RESL.

FIG. 16. DNA interaction with NFkB–RESL43 complex. Zoom bottom view represents the interaction of RESL43 at DNA binding region of NFkB. Zoom top view shows the binding interaction of DNA with the complex of NFkB–RESL43.
DNA and preventing the binding ability. These results correlate with our EMSA experimental results that RESL43 and RESL suppress the level of NFkB by preventing its ability to bind with DNA.

These in vitro and in silico findings suggest that RESL43, a novel RESL derivative, may potentially be superior to natural RESL as a candidate for a chemoprevention agent. Further insights into the structure–activity relationship of RESL43 are anticipated to endorse the development of a potential class of RESL derivatives into cancer therapeutics and their transfer into clinical application. In the immediate term, we suggest that the diacetate resveratrol derivative RESL43 warrant further evaluation in preclinical and clinical bridging studies in the near future.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

References


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