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1,2,4-Thiadiazolidine derivative inhibits nuclear transcription factor-κB and its dependent genes activation but induces apoptosis

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The 1,2,4-thiadiazolidine derivatives have been shown to be involved in several biological responses such as anti-bacterial, anti-fungal, anti-tubercular and local anaesthetic activities. In our study, we have synthesized some new 5-substitutedarylimino-2-N-substitutedphenyl-3-oxo-1,2,4-thiadiazolidine and tested for anti-inflammatory and anti-tumor activities. The 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-Oxo-1,2,4-thiadiazolidine (P_3-25) showed anti-inflammatory activity as it inhibited different inflammatory inducers mediated nuclear transcription factor kappa B (NF-κB), a key transcription factor involved in all forms of inflammation. P_3-25 inhibited TNF-induced NF-κB activation as detected by gel shift assay and dependent reporter gene expression. It inhibited IκBα degradation, IκB kinase activation and p65 nuclear translocation. P_3-25 inhibited TNF-induced Cox2 expression. It inhibited NF-κB activation in human epithelial and T cells. Unlike other substitutary derivatives, P_3-25 was a potent inducer of apoptosis as it induced cell death, caspase-dependent PARP cleavage, ROI generation and lipid peroxidation. Overall our results suggest that P_3-25 derivative exerts anti-inflammatory and anti-tumor activities, which may have a role in designing such drugs.

Key words: 1,2,4-thiadiazolidine; NF-κB; cell signaling; apoptosis; tumor

The thiazolidones and thiadiazolie have drawn considerable attention due to their varied biological and physiological activities, e.g., local anaesthetic,[1] anti-bacterial,[2] anti-fungal[2] selective Muscarinie Agonists[3] and anti-inflammatory.[4] 1,3,4-thiadiazole derivatives have also been shown to possess anti-microbial,[5] anti-convulsant,[6] carbonic anhydrase inhibitory[7] and tumor associated isozyme IX inhibitory[7] activities; 3-oxo-1,2,4-thiadizolidines have been shown to possess promising anti-fungal,[8] anti-bacterial[8] and plant growth regulator[9] activities; 1,2,4-Triazole derivatives have been shown to possess promising eosinophilia inhibitory[10] activities. The oxidative debenzylation and cyclisation[11–14] technique has been reported as a standard technique for the synthesis of N and S containing 1,2,4-thiadiazolidines. A perusal of the synthetic routes followed by earlier workers[15,16] for the synthesis of 1,2,4-thiadiazolidines, oxidative dealkylation and cyclization of isodithiobiurets and related systems, enhanced anti-fungal activities[8] associated with certain 3-oxo-1,2,4-thiadiazolidines,[8] prompted us to synthesize newer 1,2,4-thiadiazolidines by employing oxidative debenzylation technique and study their biological activities.

Nuclear transcription factor-kappa B (NF-κB), a, ubiquitous nuclear transcription factor, was first identified in 1986 by Sen and Baltimore, as reported by Baldwin.[17] Extensive research during the past few years has indicated that this factor regulates the expression of various genes that play critical roles in apoptosis, viral replication, tumor genesis, various autoimmune diseases and inflammation.[18,19] Because of its role in the pathogenesis of various diseases, NF-κB is a current target of interest used by various pharmaceutical companies.[19] As part of the stress response, NF-κB is activated in response to various inflammatory stimuli including cytokines, mitogens, bacterial products, viral proteins and apoptosis-inducing agents.[17,18] Under normal conditions, NF-κB is present in the cytoplasm as an inactive heterotrimer consisting of p50, p65 and IκBα subunits. On activation, degradation of Iκ Bα exposes nuclear localization signals on the p50–p65 heterodimer, leading to nuclear translocation and binding to a specific sequence in DNA, which in turn results in gene transcription. NF-κB, nuclear transcription factor kappa B is known to be induced by different inducers in a wide variety of cells ranging from insect to human in origin. After induction, it induces a wide variety of genes and the resulting expression of many proteins, including cyclooxygenase (Cox) 2, help in inflammation, cell growth and differentiation. So, any compound that has inhibitory activity for NF-κB may have anti-inflammatory and anti-tumor property.

Program cell death has been assayed by various methods. In our study, the effect of different derivatives were detected by cell viability assay,[20] which is reflected on the oxidative burst response. Different caspases are involved in cell death by cleaving several cellular proteins including polyADP-ribose polymerase (PARP). The lower level of generated reactive oxygen intermediates (ROI) activates NF-κB, but high ROI levels have deleterious effect by destroying cellular integrity[21] and inducing lipid peroxidation.[22] In our study, we are providing data that among several 5-substitutedarylimino-2-N-substitutedphenyl-3-oxo-1,2,4-thiadiazolidine derivatives the 5-(4-methoxyarylimino)2-N-(3,4-dichlorophenyl)-3-Oxo-1,2,4-thiadiazolidine (P_3-25) is more potent in inhibiting NF-κB activation and inducing apoptosis. Overall, the 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-Oxo-1,2,4-thiadiazolidine form, which is designated as P_3-25 throughout the study, may be helpful to design for novel anti-inflammatory and anti-tumor drugs.

Material and methods

Materials

Phorbol myristate acetate (PMA), lipopolysaccharide (*E. coli*, 055:B5), H_2O_2, N-acetyl cysteine (NAC), glycine, NaCl, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide], thiobarbituric acid, 4-methyl umbelliferyl phosphate, BSA and anti-tubulin antibody were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Penicillin, streptomycin, neomycin, RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Life Technologies (Bethesda, MD). TNF was obtained from Peprotech (Rocky Hill, NJ). IL-1 and anti-PARP antibody were obtained from Pharmingen (San Diego, CA). Antibodies against p65, IKKα, IKKβ, CRM1, IκBα, c-Rel, PARP and Cox2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Live and Dead cell assay kits were obtained from Molecular Probes (Eugene, OR). Goat-anti-rabbit IgG conjugated with HRP

Abbreviations: CE, cytoplasmic extract; FBS, fetal bovine serum; ICAM 1, intracellular adhesion molecule 1; IκBα, inhibitory subunit of kappa B; IKK, IκBα kinase; NAC, N-acetyl cysteine; NF-κB, nuclear transcription factor kappa B; MTT, 3-(4,5-Dimethyl-2-thiozolyl)-2,5-diphenyl-2H-tetrazolium bromide; NE, nuclear extract; PARP, poly-(ADP)-ribose polymerase; PMA, phorbal myristate acetate; P_3-25, 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-Oxo-1,2,4-thiadiazolidine; ROI, reactive oxygen intermediate; SEAP, secretory alkaline phosphatase; TBARS, thiobarbituric acid-reactive substance; TNF, tumor necrosis factor.

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was obtained from Bio-Rad (Richmond, CA). The plasmid constructs for NF-κB-SEAP, Cox-2-Luciferase, and dominant negative IκBα were kindly supplied by Prof. B.B. Aggarwal of the University of Texas M. D. Anderson Cancer Center (Houston, TX).

Synthesis of different derivatives of 1,2,4-thioadiazolidine and its property

The 5-(4-Methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidines were prepared by starting from 1-(4-methoxyaryl) thiocarbamide. At first, 1-(4-methoxyaryl) thiocarbamide were benzylated by benzyl chloride in ethanol solvent to form 1-(4-methoxyaryl)-2-S-benzylisocarbamides. A mixture of 1-(4-methoxyaryl)-2-S-benzylisocarbamides and 3,4-dichlorophenylisocynade in benzene medium were refluxed for 6 hr to form crude 1-(4-methoxyaryl)-5-(3,4-dichlorophenyl)-2-S-benzyliso-4-biurets, which was subsequently purified by recryrstallization from ethanol. Finally, 1-(4-methoxyaryl)-5-(3,4-dichlorophenyl)-2-S-benzyliso-4-biurets was made into a paste with chloroform and oxidized with molecular bromine to form crude 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine which was purified by recrystalization from ethanol.

5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine

The following values applied: yield 70.4%, m.p-265°C; $C_{15}H_{11}N_3SO_2Cl_2$ (368), found (%): C(49.08) H(2.83) N(11.27) S(8.83) calculated (%): C(48.91) H(2.98) N(11.41) S(8.69); IR (νin cm^{-1}): 3292(NH),1649(C=O),1577(C=N),660(C-Cl); ^{1}H-NMR (in DMSO-d_6/TMS) δ(ppm): 3.36(3H,OCH_3), 7.30 (1H,NH), 7.78(7H,Ar-H); ^{13}C-NMR (in DMSO-d_6) δ(ppm): 168.82(C=N), 163.76(C=O), 119.84–138.31 (aromatic carbon), 56.51(OCH_3) and Mass spectra (m/z): 368(M^+).

Cell lines

The cell lines used in our study were as follows: U-937 (histiocytic lymphoma), HeLa (epithelial cells), Jurkat (T cell) and H4 (glial cells). These cells were obtained from American Type Culture Collection (Manassas, VA). Cells were culture 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).

NF-κB activation assay

To determine NF-κB activation, EMSA were conducted essentially as described.[20] Briefly, 2×10^6 cells, after different treatments, were washed with cold PBS and suspended in 0.4 ml of hypotonic cytoplasmic extraction buffer. The cells were allowed to swell on ice for 15 min, after which 12.5 μl of 10% Nonidet P-40 was added. The tube was then vigorously mixed on a vortex machine for 10 sec, and the homogenate was centrifuged for 30 sec in a microfuge. The nuclear pellet was resuspended in 25 μl of ice-cold nuclear extraction buffer, and the tube was incubated on ice for 30 min with intermittent mixing. The tube was then centrifuged for 5 min in a microfuge at 4°C, and the supernatant (nuclear extract) was either used immediately or stored at −70°C for later use. Eight micrograms of nuclear extract proteins were incubated with ^{32}P end-labeled 45-mer double-stranded NF-κB oligonucleotide of HIV-LTR, 5′-TTG TTA CAA GGG ACT TTC CGC TGG GGA CTT TCC AGG GAG GCG TGG-3′ (bold indicates NF-κB binding site) for 30 min at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. The specificity of binding was examined by competition with unlabeled and ^{32}P end labeled mutant oligonucleotides. Visualization of radioactive bands was done in a PhosphorImager (Fuji, Japan) using Image Reader software.

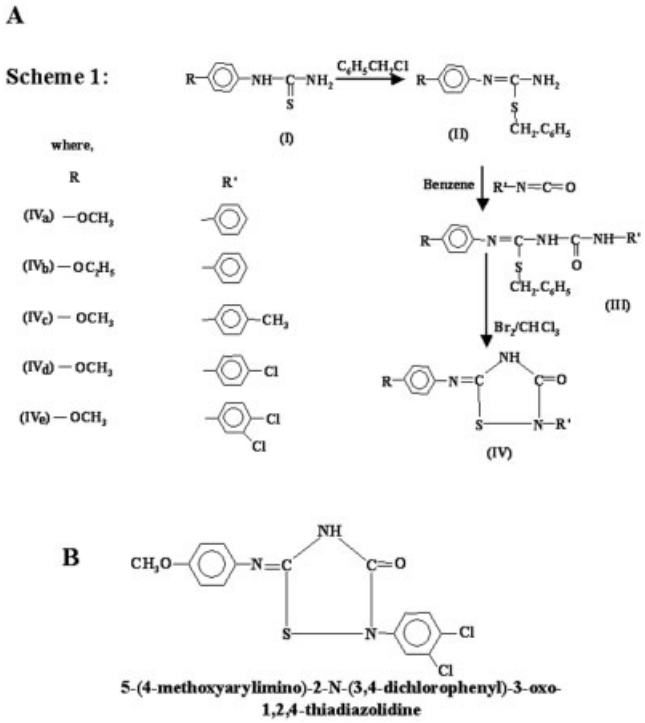


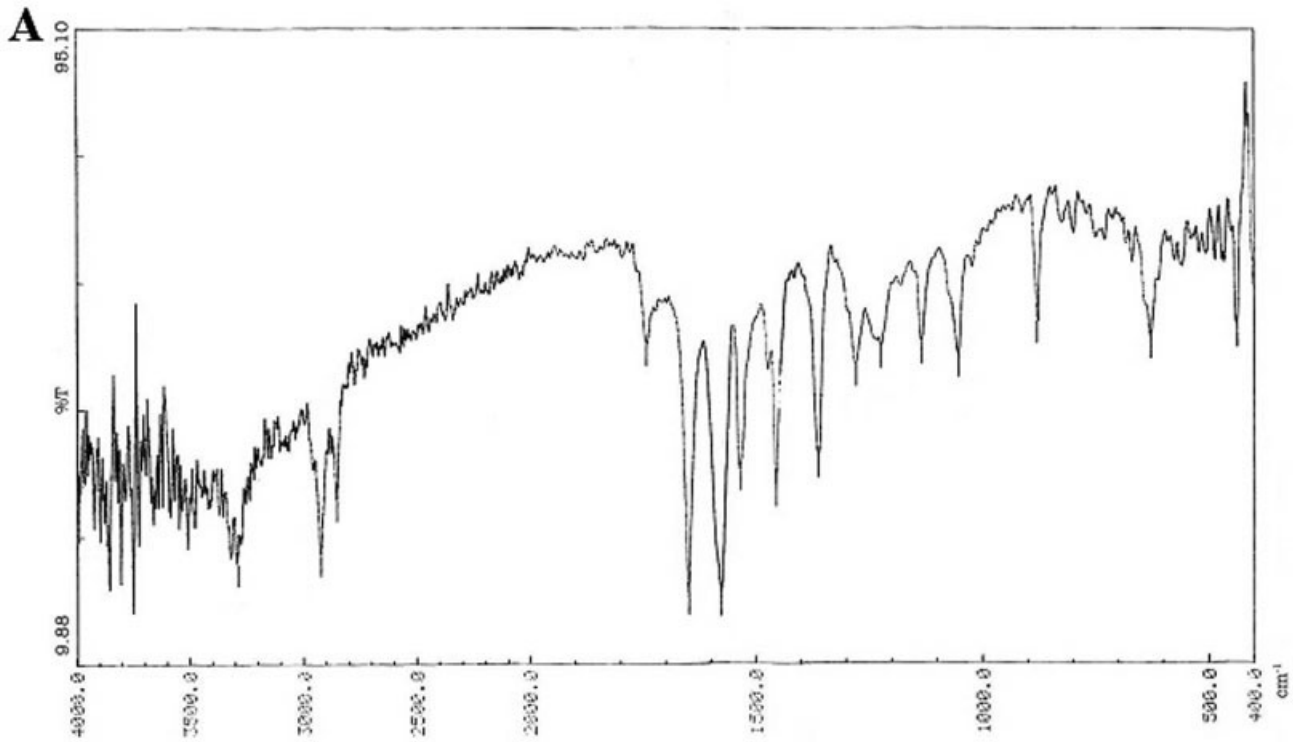
FIGURE 1 – (*a*) Schematic diagram for the synthesis of 1,2,4-thiadiazolidine derivatives. (*b*) Structure of 5-(4-Methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine (P_3-25).

NF-κB-dependent reporter gene transcription assay

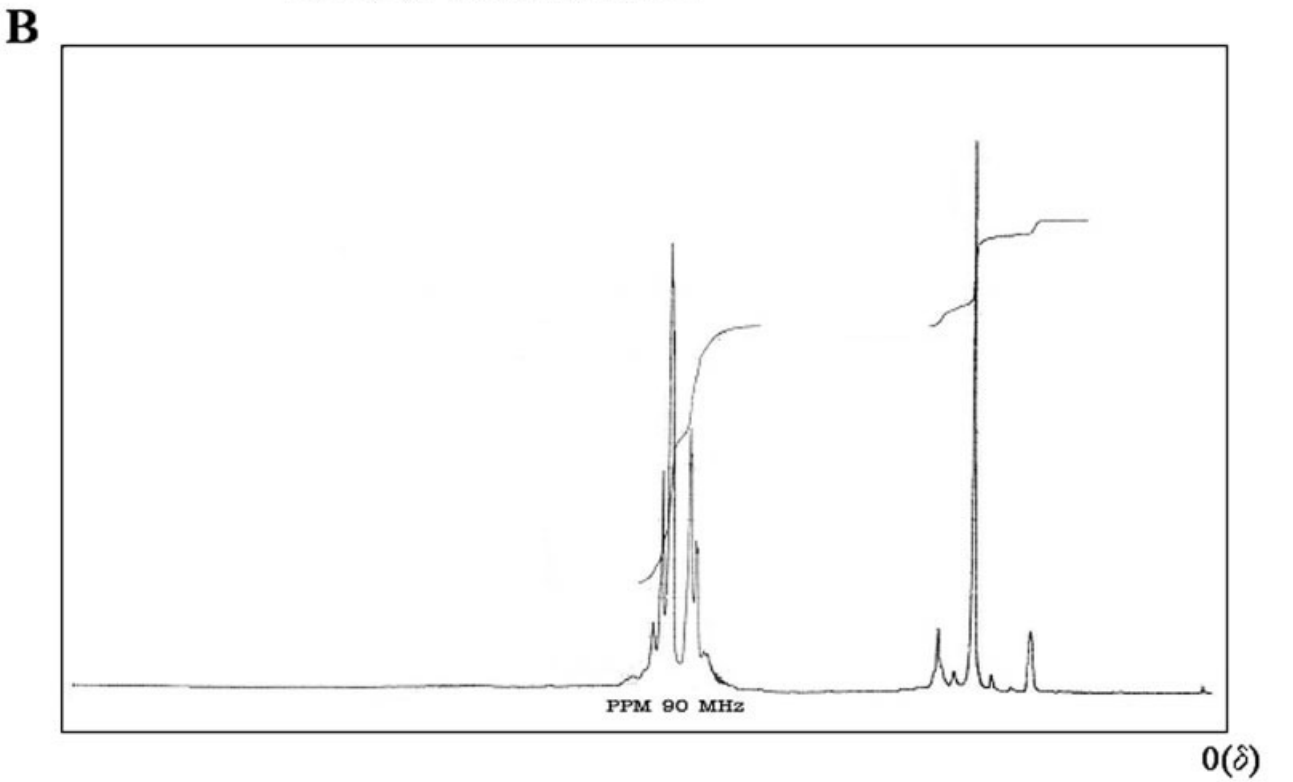
The 1,2,4-thiadiazolidine derivatives-induced NF-κB-dependent reporter gene transcription was measured as previously described.[23] Briefly, U-937 cells were transiently transfected by the calcium phosphate method with 1 ml medium containing reporter genes NF-κB promoter DNA linked to the heat-stable secretory alkaline phosphatase (SEAP) (0.5 μg) and β-galactosidae (0.5 μg) with or without dominant negative IκBα (0.5 μg) construct. The total amount of DNA was maintained at 3 μg by the addition of the control plasmid pCMVFLAG1 DNA. After 6 hr of transfection cells were washed and culture for 12 hr. Cells were treated with different concentrations of 1,2,4-thiadiazolidine derivatives for 3 hr and then stimulated with TNF (0.1 nM) for 12 hr. Cell culture-conditioned medium was harvested, and 25 μl was analyzed for alkaline phosphatase activity essentially as per the CLONTECH protocol (Palo Alto, CA) using 4-methylumbelliferyl phosphate as substrate for SEAP and reported as fold activation with respect to control vector-transected cells. This reporter system was specific, because TNF-induced NF-κB SEAP activity was inhibited by overexpression of IκBα mutants lacking either Ser^{32} or Ser^{36}.[23] For transfection control, cells were cotransfected with β-galactosidase and the activity was assayed from each transfection.

Cox2-dependent reporter gene transcription

The effect of 1,2,4-thiadiazolidine derivatives on TNF-induced Cox2-dependent luciferase reporter gene expression was carried out as described previously.[24] U-937 cells were transiently transfected by the calcium phosphate method with 1 ml medium containing Cox2-Lucifearse (0.5 μg) and β-galactosidase (0.5 μg) construct. The total amount of DNA was maintained at 3 μg by the addition of the control plasmid pCMVFLAG1 DNA. After 6 hr of transfection cells were washed and culture for 12 hr. Cells were treated with different concentrations of 1,2,4-thiadiazolidine derivatives for 3 hr and then stimulated with TNF (0.1 nM) for 12 hr. The cell pellets were collected and extracted with lysis buffer (part



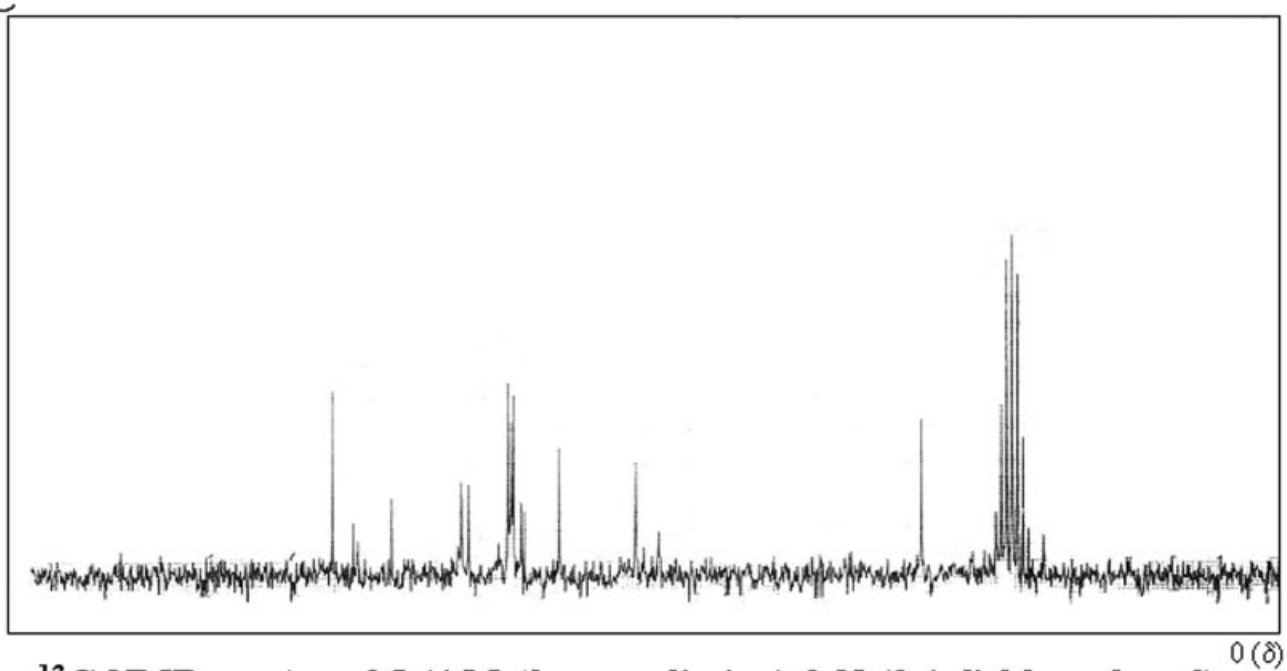
IR spectra of 5-(4-Methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine



^{1}H-NMR spectra of 5-(4-Methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine

Figure 2 – (*a*) Infrared (IR) spectroscopy of 5-(4-Methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine (P_3-25). (*b*) ^{1}H-NMR spectroscopy of P_3-25. (*c*) ^{13}C-NMR spectroscopy of P_3-25. (*d*) Mass spectroscopy of P_3-25.

C



^{13}C-NMR spectra of 5-(4-Methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine

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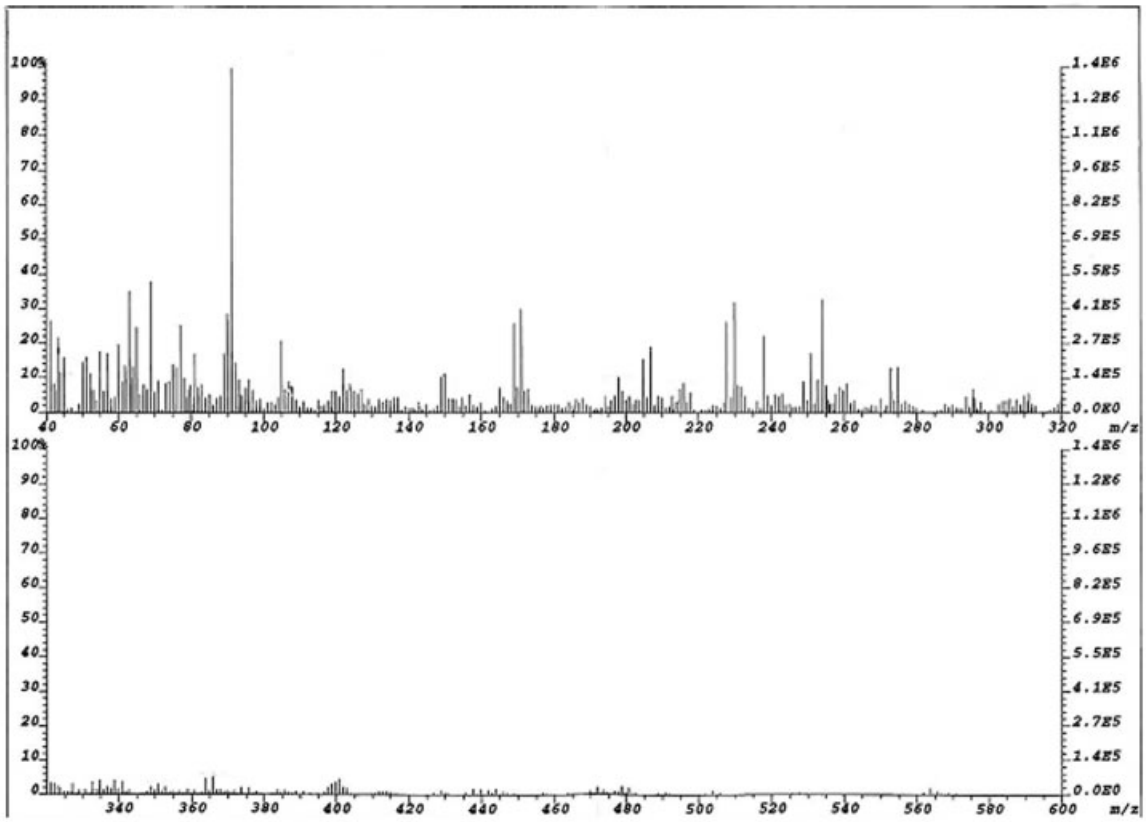

Mass spectra of 5-(4-Methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine

FIGURE 2 – CONTINUED.

of Luciferase assay kit from Promega, Madison, WI). Cell extracts were incubated with the firefly luciferin (substrate, Promega). Light emission was monitored with a Luminometer and values were calculated as fold of activation over vector-transfected value. The activity of β-galactosidase was assayed from the same extracts.

Western blot for IκBα and p65

To determine the levels of IκBα and p65, cytoplasmic extracts, prepared from cells pre-treated with 100 nM of P_3-25 for 3 hr and then simulated with TNF (0.1 nM) for different times were resolved on 9% SDS-polyacrylamide gel.[20] Western blot was done using anti-IκBα, and -p65 antibody and detected by chemiluminescence (ECL, Amersham, Arlington Heights, IL).

IKK assay

The IKK assay was performed by a method described previously.[24] Briefly, IKK complex from whole-cell extract (300 μg) was precipitated with anti-IKKα and IKKβ antibodies (1 μg each), followed by incubation with protein A/G-Sepharose beads (Pierce, Rockford, IL). After a 2 hr incubation, the beads were washed with lysis buffer and then assayed in kinase assayed using 2 μg of substrate GST-IκBα (aa1-aa54).

Cytotoxicity assay (MTT assay)

The cytotoxicity was assayed by the MTT dye uptake.[20] Briefly, U937 cells (10^4 cells/100 μl/well of 96-well plate) were incubated with 100 nM of each of different 1,2,4-thiadiazolidine derivatives for 6 hr and then treated with different chemotherapeutic agents or inducers apoptosis for 72 hr at 37°C. Thereafter, 25 μl of MTT solution (5 mg/ml in PBS) was added to each well. After 2 hr incubation at 37°C, 0.1 ml of the extraction buffer (20% SDS and 50% dimethylformamide) was added. After an overnight incubation at 37°C, the absorbance was read at 570 nm using a 96-well multiscanner autoreader (Bio-Rad), with the extraction buffer as a blank.

Cytotoxicity assay (live and dead assay)

The cytotoxic effect of 1,2,4-thiadiazolidine derivatives was also determined by the Live/Dead assay (Molecular Probes, Eugene, OR).[25] Briefly, 1×10^5 cells were incubated with 100 nM of those derivatives for 24 hr at 37°C. Cells were stained with Live/Dead reagent (5 μM ethidium homodimer, 5 μM calcein-AM) and then incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan).

Determination of lipid peroxidation

Different 1,2,4-thiadiazolidine derivatives-induced lipid peroxidation was determined by detection of thiobarbituric acid-reactive malondialdehyde (MDA), an end product of the peroxidation of polyunsaturated fatty acids and related esters, as described.[26,27] After treatment cells (3×10^6/ml) were extracted by 3 cycles of freeze thawing in 200 μl water. After protein determination, 500 μg protein (in 0.1 ml) was added to 0.8 ml assay mix containing 0.4% (w/v) thiobarbituric acid, 0.5% (w/v) SDS and 9.4% (v/v) acetic acid (pH 3.5). After incubation for 1 hr at 95°C, samples were cooled to room temperature, centrifuged at 14,000*g* for 10 min, and the absorbance of the supernatants was read at 532 nm. Results were expressed as a percentage of thiobarbituric acid-reactive substances above control values. Untreated cells showed 0.571 ± 0.126 nmol of MDA equivalents/mg protein.

Measurement of ROI

The production of ROI on treatment of cells with different 1,2,4-thiadiazolidine derivatives was determined by flow cytometry as described.[28] Briefly U-937 cells (5×10^5) were incubated with different concentrations of 1,2,4-thiadiazolidine derivatives for 3 hr at 37°C. Cells were then stimulated with 1 nM TNF for 2 hr, washed with D-PBS and resuspended in 1 ml D-PBS. To detect ROI production, cells were exposed to dihydrorhodamine 123 (5 mM stock in DMSO) at a final concentration of 1 μM for 1 hr at 37°C with moderate shaking (100 rpm) and then washed with D-PBS 3 times and resuspended in 1 ml D-PBS. Rhodamine 123 fluorescence intensity resulting from dihydrorhodamine 123 oxidation was measured by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) with excitation at 488 nm and was detected between 515 and 550 mm. Data analysis was performed using LYSYSII software (Becton Dickinson).

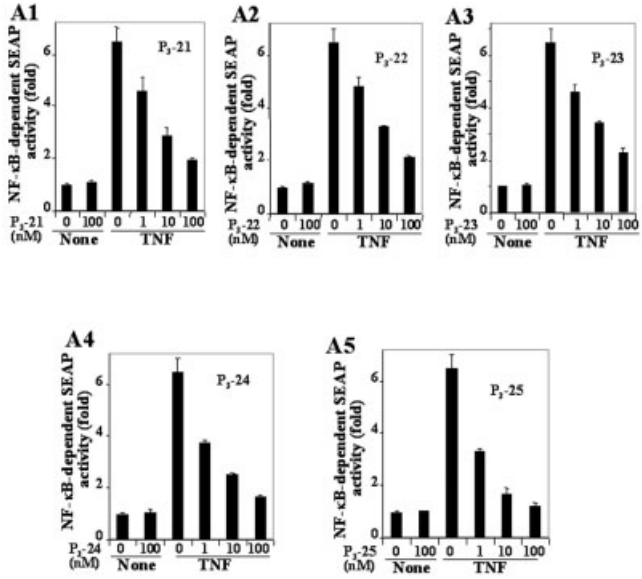

FIGURE 3 – Effect of 1,2,4-thiadiazolidine derivatives on TNF-induced NF-κB-dependent SEAP activation. U937 cells, transiently co-transfected with NF-κB-containing plasmid linked to the *SEAP* gene and β-galactosidase were treated with different concentrations of 1,2,4-thiadiazolidine derivatives (P_3-21 to P_3-25) for 3 hr (*a*1–*a*5). Cells were then stimulated with 0.1 nM TNF for 12 hr. Culture supernatants were assayed for secreted alkaline phosphatase activity as described in Material and Methods. Results are expressed as fold activity over the nontransfected control.

Results

In our study, we examined the effect of different 1,2,4-thiadiazolidine derivatives, which were chemically synthesized in the laboratory. All these derivatives were dissolve in DMSO at 10 mM concentration. Further dilution was carried out in medium. Most of the experiments were carried out using U937 cells because their response to NF-κB activation by various stimuli has been well characterized in our laboratory. At the concentration of 1,2,4-thiadiazolidine derivatives and duration of exposure employed in these studies, there was no effect on cell viability as detected by the trypan blue dye exclusion method (data not shown). We used P_3-25, one of the derivatives of 1,2,4-thiadiazolidine, for most of the study.

Synthesis of different derivatives of 1,2,4-thioadiazolidine

Synthesis of 5-substitutedarylimino-2-N-substitutedphenyl-3-oxo-1,2,4-thiadiazolidines (IVa–e) (Fig. 1*a*, Scheme 1) from the related 1-substitutedaryl-5-substitutedphenyl-2-S-benzyliso-4-biurets (IIIa–e) by oxidative debenzylation technique. The preparation of compounds 1-substitutedaryl-5-substitutedphenyl-2-S-benzyliso-4-biurets (IIIa–e) was achieved[13,15] by the condensation of related S-benzylisocarbamides (IIa–e) and substitutedphenylisocyanate in benzene medium at its refluxing temperature for 6 hr (as indicated in Scheme 1). The structure of compound (IVa–e) was confirmed by element analyses, IR (Fig. 2*a*), ^{1}H-NMR (Fig. 2*b*), ^{13}C-NMR (Fig. 2*c*) and mass spectra (Fig. 2*d*).

1,2,4-thioadiazolidine derivatives blocks TNF-induced NF-κB-dependent reporter gene expression

To detect the role of different derivatives of 1,2,4-thioadiazolidine on NF-κB-dependent reporter gene expression, U937 cells were transiently cotransfected with NF-κB-containing plasmid linked to the SEAP gene and β-galactosidase with or without dominant negative IκBα plasmids. Cells, treated with different concentrations of thiocarbamide derivatives for 3 hr and then stimulated with 0.1 nM TNF for 12 hr. The SEAP activity was assayed in culture supernatant. Results are expressed as fold activity over the nontransfected control. The results showed in Figure 3 that different derivatives (Fig. 3*a*1–*a*5) decreased TNF-induced SEAP activity in a dose-dependent manner. Additionally, P_3-25 was more effective in inhibiting TNF-induced NF-κB dependent SEAP gene expression. IκBα-DN transfected cells showed no induction of SEAP activity by TNF (data not shown). The β-galactosidase activity from cell extracts showed an almost similar reduction of absorbance (as per Promega protocol) at 420 nm (data not shown), suggesting the transfection control was successful for each treatment.

P_3-25 inhibits TNF-induced NF-κB activation

U937 cells (2×10^6/ml) were pretreated with different concentrations of P_3-25 for 3 hr and then stimulated with TNF (0.1 nM) for 1 hr. Cells were washed and nuclear extracts were prepared. Eight micrograms of nuclear extract proteins were assayed for NF-κB by EMSA. As shown in Fig.4*a*, TNF induced NF-κB activation potently and P_3-25 inhibited this activation in a dose-dependent manner; full inhibition occurred at 0.1 μM and at this concentration even higher concentration did not activate NF-κB. Most of the study was carried out with 0.1 μM concentration of P_3-25.

P_3-25 inhibits TNF-induced IκBα degradation, IKK activation, and nuclear translocation of p65

The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα. To determine whether the inhibitory action of P_3-25 was due to its effect on IκBα degradation, the cytoplasmic levels of IκBα protein were examined by Western blot analysis. Both untreated and P_3-25-pretreated cells were incubated with TNF (0.1 nM) for different times and then cytoplasmic extracts were used to assay IκBα by Western blot. Upon treatment with TNF the level of IκBα decreased within 10 min and then reappeared slowly from 30 min, indicating degradation followed by resynthesis of IκBα (Fig. 4*b*1). P_3-25 pre-treated cells exhibited a sustained IκBα band at all time points indicating that P_3-25 treatment leads to inhibition of TNF-

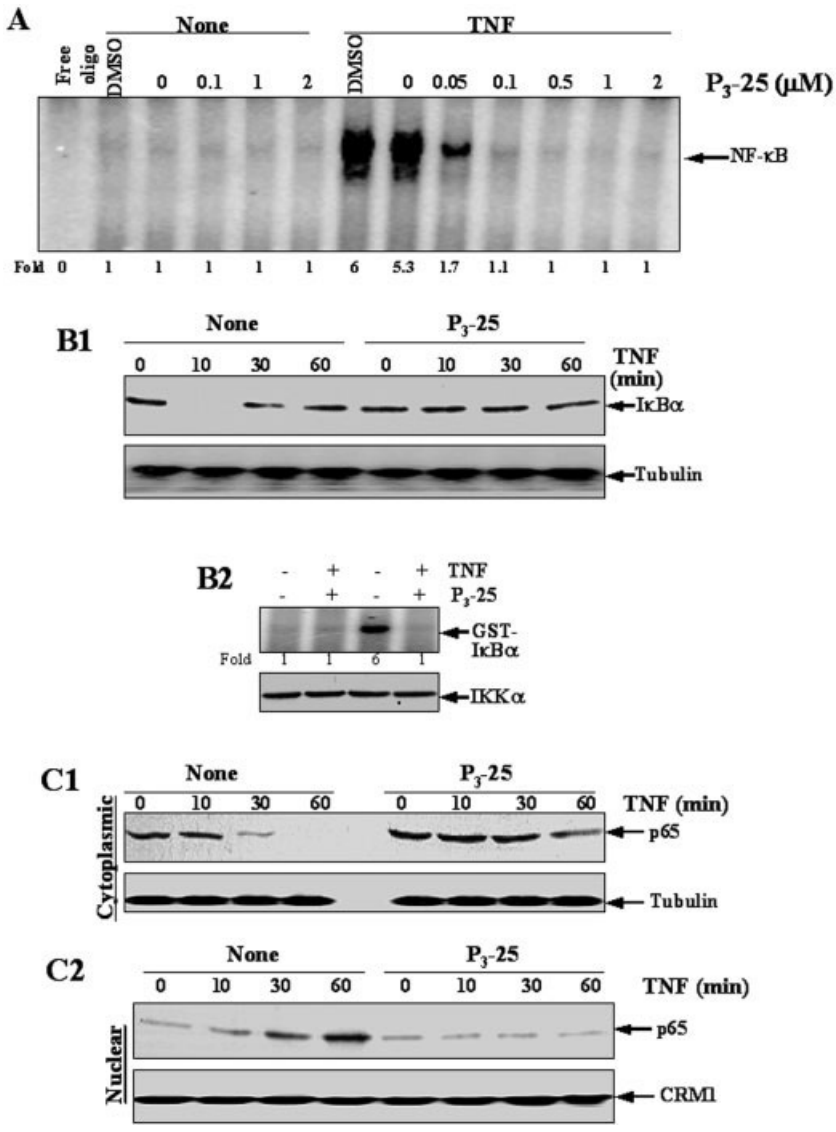

FIGURE 4 – (*a*) Effect of P_3-25 for the inhibition of TNF-dependent NF-κB activation. U937 cells were preincubated for 3 hr with different concentrations (0–2 μM) of P_3-25, followed by 1 hr incubation with 100 pM TNF. After these treatments, nuclear extracts were prepared and then assayed for NF-κB, as described in Material and Methods. (*b*1) Effect of P_3-25 on TNF-induced IκBα degradation. Cells were incubated at 37°C with 0.1 μM P_3-25 for 3 hr and then stimulated with 100 pM TNF for different times. Cytoplasmic extracts were prepared and 50 μg proteins were analyzed in 9% SDS-PAGE and assayed for IκBα by Western blot analysis. The same blot was probed with anti-tubulin antibody. (*b*2) Effect of P_3-25 on TNF-induced IKK activation. Cells were treated with 0.1 μM P_3-25 for 3 hr and then stimulated with 100 pM TNF for 15 min. Then cell extracts were used to assay IKK as described in Material and Methods. (*c*) Effect of P_3-25 on TNF-induced p65 translocation. Cells were incubated at 37°C with 0.1 μM P_3-25 for 3 hr and then stimulated with 100 pM TNF for different times. Cytoplasmic and nuclear extracts were then used to detect the p65 level. The blots were reprobed with tubulin and CRM1 antibodies, respectively.

induced IκBα degradation. Thus, these results strongly suggest that P_3-25 blocks TNF-mediated degradation of IκBα.

Since IKK activation is necessary for TNF-induced IκBα phosphorylation and degradation, the effect of P_3-25 on IKK activation was examined. U-937 cells were pre-treated with P_3-25 (0.1 μM) for 3 hr and then stimulated with 0.1 nM TNF for different times. The cell extracts were prepared and IKK activation was detected *in vitro* using GST-IκBα as substrate protein as described in Material and Methods. The GST-IκBα specific signal was increased to 6-fold with TNF incubation, whereas P_3-25 pretreatment completely suppressed the band (Fig.4*b*2), indicating P_3-25's role on suppression of TNF-induced IKK activation. Fifty micrograms of extract proteins were analyzed in SDS-PAGE (9%) and probed with anti-IKKα antibody. The intensity of bands was equal in all lanes, suggesting equal expression of IKKα.

We performed Western blot analysis to determine whether P_3-25 affects the TNF-induced nuclear translocation of the p65 subunit of NF-κB. Upon TNF treatment, the p65 level was decreased in the cytoplasm and increased in the nucleus with time, whereas in P_3-25-pretreated cells, TNF was unable decrease the p65 level in the cytoplasm or unable to increase the p65 level in nucleus with time (Fig. 4*c*1,*c*2).

P_3-25 blocks PMA-, LPS-, TNF- and IL-1-mediated activation of NF-κB

Various tumor promoters and inflammatory agents, including PMA, H_2O_2, LPS, IL-1 and TNF[18] by different signal transduction pathways,[29–31] activate NF-κB. U937 cells were treated with 100 nM P_3-25 for 3 hr and then stimulated with PMA (25 ng/ml), serum activated LPS (100 ng/ml), H_2O_2 (250 μM), IL-1 (100 ng/ml) and TNF (100 pM) for 1 hr. Nuclear extracts were prepared and assayed for NF-κB as described before. We found that these agents activated NF-κB and that P_3-25 completely blocked the activation of NF-κB induced by all inducers except H_2O_2 (Fig. 5*a*). These results suggest that P_3-25 may act at a step in which all these agents converge in the signal transduction pathway leading to NF-κB activation. H_2O_2-mediated NF-κB activation may follow different pathway, which P_3-25 is unable to inhibit

Supershift and specificity of NF-κB band

To detect the composition and specificity of the retarded band visualized by EMSA, nuclear extracts from TNF-activated cells were incubated with antibodies (Abs) p50 (NF-κBI), p65 (Rel A), or in combination, 50-fold excess of cold NF-κB, and labeled mutated NF-κB oligonucleotides then conducted EMSA. Abs to either subunit of NF-κB shifted the band to a higher m.w. (Fig. 5*b*), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. The irrelevant Abs such as anti-c-Rel or anti-cyclin D1 had no effect on the mobility of NF-κB. The complex completely disappeared in presence of cold NF-κB or without any binding with mutant oligonucleotide indicating the specificity of NF-κB.

P_3-25 inhibits TNF-induced Cox2 expression

Since P_3-25 inhibited TNF-induced different biological responses, the expression of NF-κB regulated genes, such as Cox2, was examined. U937 cells were treated with different concentrations of P_3-25 for 3 hr and then stimulated with 100 pM TNF for 12 hr. Then 100 μg cell extract proteins were analyzed in 9% SDS-PAGE to detect Cox2 by Western blot analysis. TNF induced Cox2 (Fig. 6*a*) expression was decreased with increased concentrations of P_3-25 treatment. P_3-25 alone did not show any induction of Cox2 at 100 nM concentration. The Cox2 expression was observed in a time-dependent manner with TNF (100 pM) and P_3-25 inhibited completely the Cox2 expression at any time of TNF incubation (Fig. 6*b*). Upon reprobing the gels with anti-tubulin antibody, we found that the band intensities in all lanes were uniform. indicating equal loading of extracted protein in the lanes.

Although we have shown that P_3-25 blocks the Cox2 expression, the dependent gene transcription was also assayed. U-937 cells transiently cotransfected with the *Cox2-Luciferase* reporter and *β-galactosidase* constructs and then treated with different concentrations of different derivatives of 1,2,4-thioadiazolidine for 3 hr. Cells were then stimulated with TNF (0.1 nM) for 12 hr. An almost 7-fold increase in luciferase activity over the vector control was noted upon stimulation with TNF (Fig. 6*c*). TNF-induced luciferase activities were decreased with increased concentrations of different derivatives. P_3-25 was shown to be a more potent inhibitor for downregulation of Cox2-dependent luciferase than other derivatives. The β-galactosidase activity from cell extracts showed almost similar reduction of absorbance (as per Promega protocol) at 420 nm (data not shown), suggesting the transfection control for each treatment.

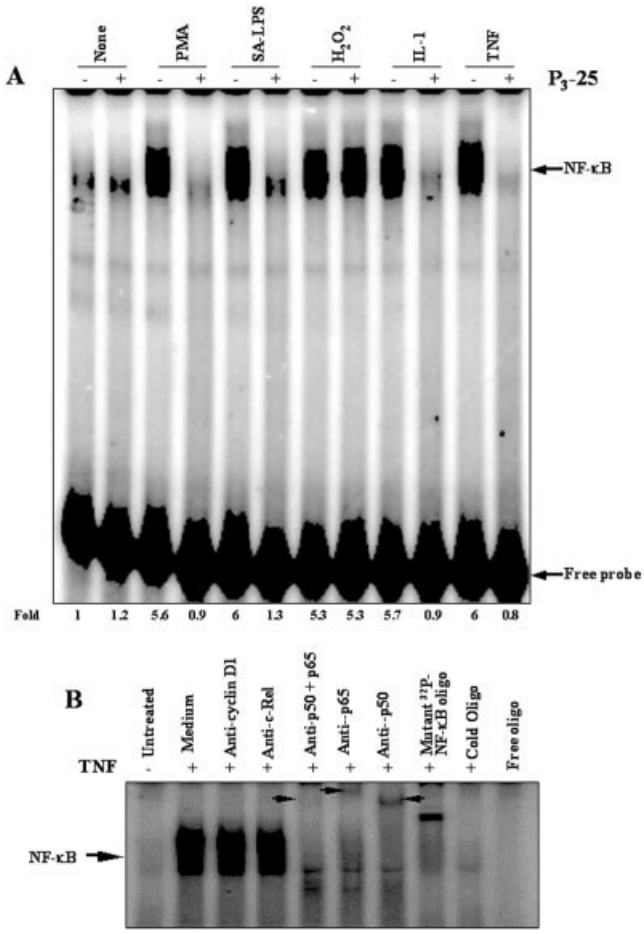

FIGURE 5 – Effect of P_3-25 on different activators (PMA, serum-activated LPS, H_2O_2, IL-1 and TNF) induced NF-κB activation. U937 cells were preincubated for 3 hr at 37°C with 100 nM P_3-25, followed by PMA (25 ng/ml), serum-activated LPS (100 ng/ml), H_2O_2 (250 μM), IL-1 (100 ng/ml), and TNF (0.1 nM) for 1 hr and then tested for NF-κB activation, as described in Material and Methods. (*b*) Nuclear extracts were prepared from untreated or TNF-treated U937 cells, incubated for 15 min with different Abs, cold NF-κB or labeled mutant NF-κB oligonucleotides and then assayed for NF-κB, as described in Material and Methods.

Inhibition of NF-κB activation by P_3-25 is cell type specific

Since NF-κB activation pathways differ in different cell types,[29] we therefore studied whether P_3-25 affects other cell type as well. It has been demonstrated that distinct signal transduction pathways could mediate induction in human glial, epithelial or T cells. All the effects of P_3-25 described above were conducted with U937 cells, a myeloid cell line. We found that P_3-25 blocks TNF-induced NF-κB activation in Jurkat (human T-lymphocytic) (Fig. 7*a*) and HeLa (human epithe-

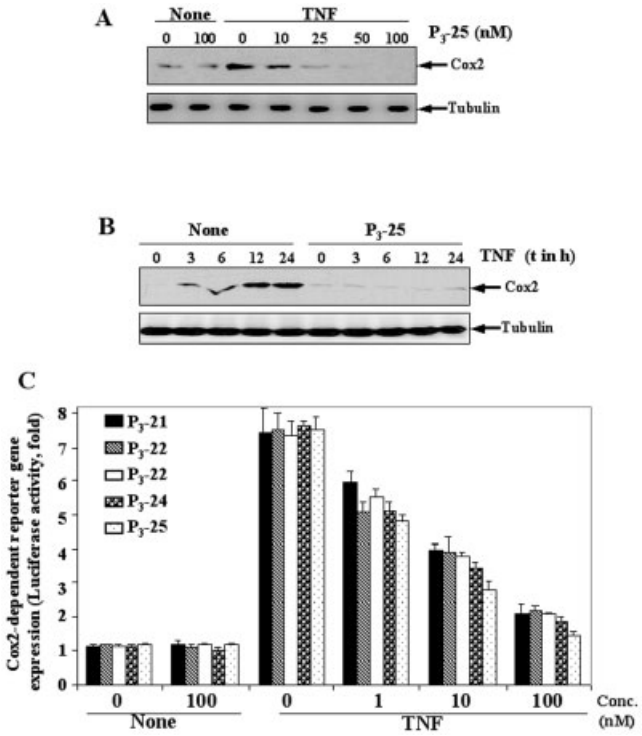

FIGURE 6 – Effect of P_3-25 on TNF induced Cox2 expression. Cells were treated with either different concentrations of P_3-25 for 3 hr and then stimulated with TNF 100 pM for 12 hr (*a*) or 0.1 mM P_3-25 for 3 hr and then stimulated with 100 pM TNF for different time (*b*) at 37°C in a CO_2 incubator. Cox2 was detected from cell extract proteins (100 μg) by Western blot analysis. The same blow was reprobed with anti-tubulin antibody. (*c*) U-937 cells were transfected with the Cox2-Luciferase expression vector and β-galactosidase gene. After 12 hr of transfection, cells were treated with different concentrations of 1,2,4-thiadiazolidine derivatives for 3 hr and then stimulated with 0.1 nM TNF for 12 hr. The luciferase and β-galactosidase enzymes activity was measured. Luciferase activity was indicated as fold of activation above vector transfected cells.

lial) (Fig. 7*b*) cells but not in H4 (human glial cells) (Fig.7*c*). This results suggests that the cell-specific interaction of P_3-25.

1,2,4-thiadiazolidine derivatives induce PARP cleavage, cell death and potentiate TNF-induced cytotoxicity

Among all the inducers, TNF is one of the most potent inducers of apoptosis.[32] Whether 1,2,4-thiadiazolidine derivatives modulate TNF-induced apoptosis was investigated. U937 cells, treated with 100 nM of different derivatives of 1,2,4-thiadiazolidine for 3 hr, were incubated with 0.1 nM of TNF for 72 hr and then examined for cytotoxicity by the MTT method. Results in Figure 7*a* show that the cytotoxic effect of TNF in U937 cells was about 35%. Different derivatives alone induced cytotoxicity between 50 to 65%. TNF further increased cytotoxicity by approximately 20% more in addition of its own cytotoxic effect. The result suggests that TNF induced cytotoxicity is potentiated by different 1,2,4-thiadiazolidine derivatives. Because the cytotoxic effect of 1,2,4-thiadiazolidine derivatives occurred alone or in combination with TNF, we also examined the effect of P_3-24- and P_3-25-induced caspase activation in the form of PARP protein cleavage. As shown in Figure 7*b*, both P_3-24- and P_3-25 induced cleavage of PARP in a dose-dependent manner. This result provides further evidence that 1,2,4-thiadiazolidine derivatives induce apoptosis. NF-κB is known to be involved in apoptosis and since 1,2,4-thiadiazolidine derivatives were found to downregulate NF-κB, we

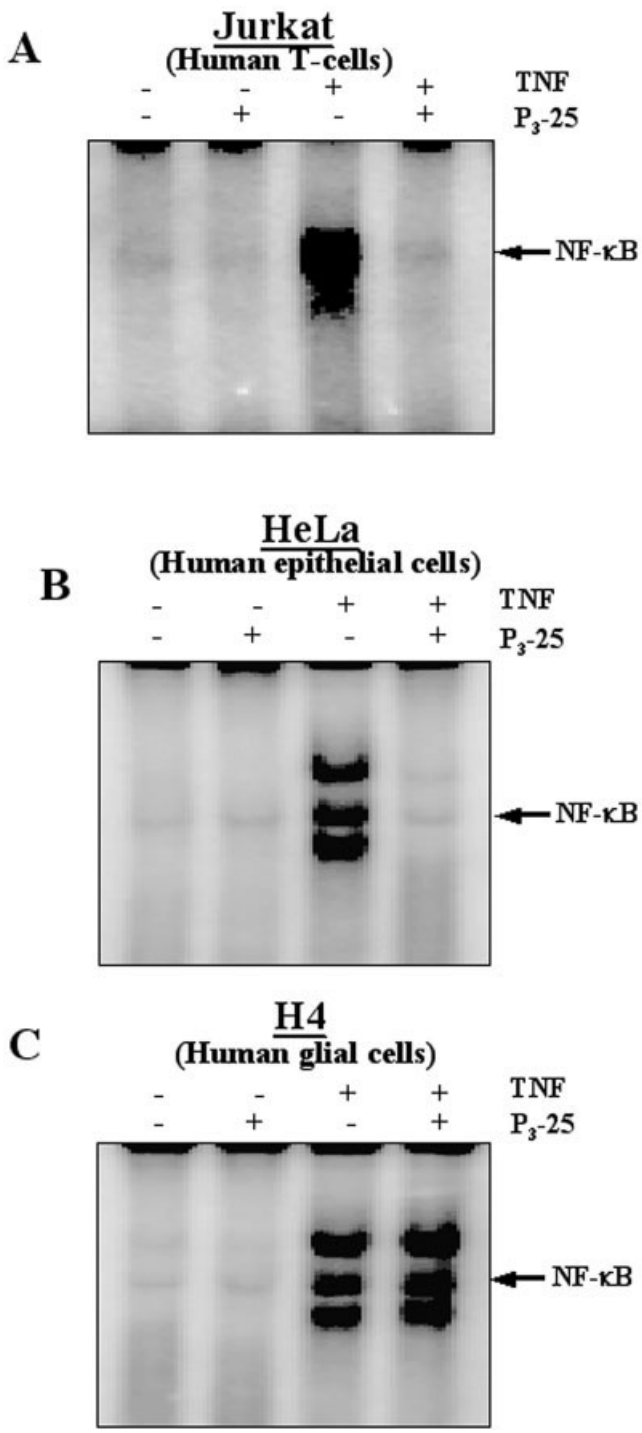

FIGURE 7 – Effect of P_3-25 on activation of NF-κB induced by TNF in different cell lines. Human Jurkat and H4, and HeLa cells were treated with 100 nM P_3-25 for 3 hr and then stimulated at 37°C for 1 hr with 100 pM TNF. After these treatments, nuclear extracts were prepared and then assayed for NF-κB.

hypothesized that P_3-25 pretreatment might potentiate the apoptotic action. U937 cells were incubated with different concentrations of P_3-25 for 72 hr and checked for cell viability by fluorometric Live and Dead cell assay as described in Material and Methods. Results indicate that P_3-25 induced cell death in a concentration dependent manner (Fig. 7*c*). Our results thus indicate that P_3-25 induces cell death.

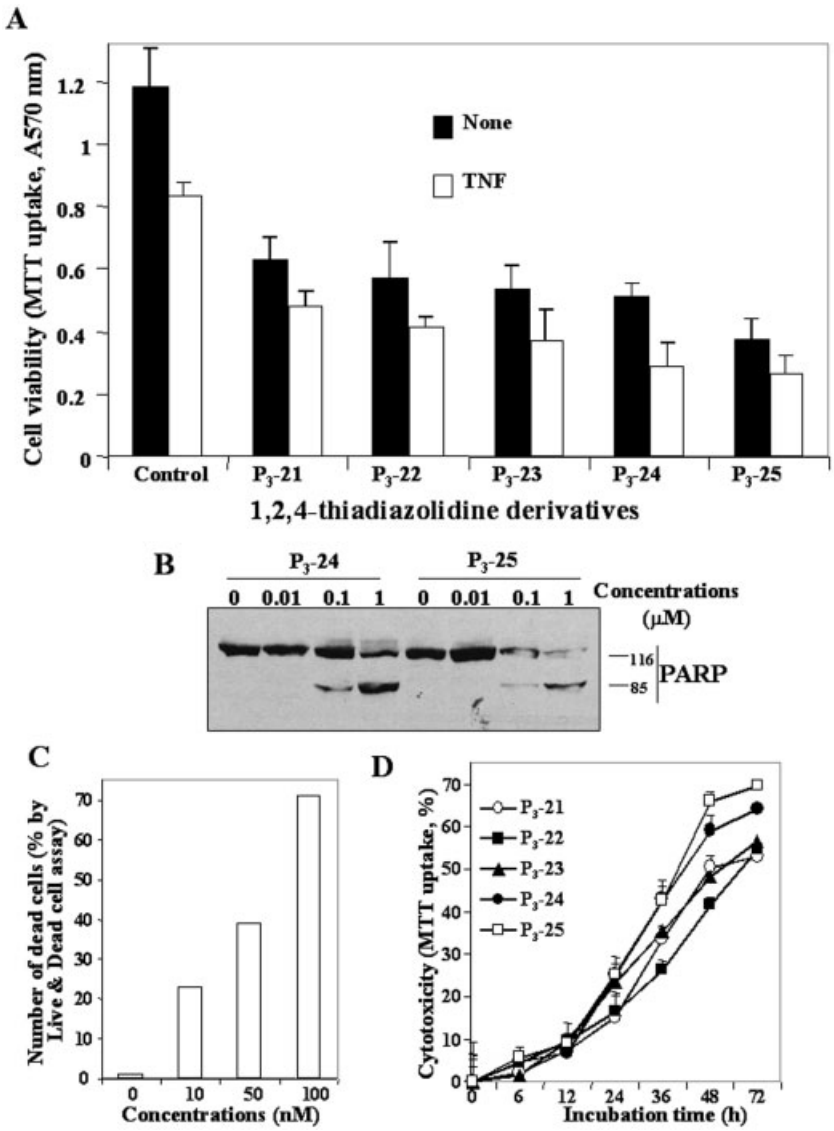

FIGURE 8 – (*a*) Effect of different 1,2,4-thiadiazolidine derivatives on TNF-induced cytotoxicity. U937 cells, untreated or pretreated with 100 nM of different 1,2,4-thiadiazolidine derivatives for 3 hr at 37°C were incubated with 100 pM TNF for 72 hr at 37°C, in a CO_2 incubator. Then MTT assayed and results were indicated in mean OD of triplicate assays. (*b*) Effect of P_3-24 and P_3-25 on PARP cleavage. U937 cells were treated with different concentrations of P_3-24 and P_3-25 for 24 hr at 37°C. Then cell extracts proteins (50 μg) were analyzed by Western blot to detect PARP using anti-PARP MAb. (*c*) Effect of P_3-25 on cell viability. Cells were pretreated different concentrations of P_3-25 for 72 hr. The cell viability was assayed by "Live/ Dead cell assay" and represented in % of dead cells. (*d*) Effect of 1,2,4-thiadiazolidine derivatives on cytotoxicity. Cells, treated with 0.1 μM of each derivatives as indicated times were assayed for cytotoxicity by MTT method. Result represented in % of cytotoxicity is 1 of the 3 independent experiments.

1,2,4-thiadiazolidine derivatives induce cytotoxicity in a time-dependent manner

To detect time-dependent cytotoxicity induced by different 1,2,4-thiadiazolidine derivatives, cells were incubated with 100 pM of each derivatives for different times at 37°C and cytotoxicity was assayed by the MTT method. All these derivatives induced cell killing in a time-dependent manner and P_3-25 showed about 70% cytotoxicity at 48 and 72 hr of incubations (Fig. 8*d*).

1,2,4-thiadiazolidine derivatives potentiate TNF-induced ROI generation and lipid peroxidation

Whether 1,2,4-thiadiazolidine derivatives mediate its effects through suppression of ROI production has been examined by flow cytometry. As shown in Figure 9*a*, TNF treatment led to induction of ROI generation. Different 1,2,4-thiadiazolidine derivatives alone induced ROI generation in a dose-dependent manner and TNF-induced ROI generation further potentiated at any concentration of derivatives (Fig. 9*a*).

Since 1,2,4-thiadiazolidine derivatives induced ROI generation alone in a dose-dependent manner, we also examined the effect of 1,2,4-thiadiazolidine derivatives on lipid peroxidation in term of malondialdehyde production. Results in Figure 9*b* showed that different 1,2,4-thiadiazolidine-derivatives induced lipid peroxidation in U937 cells were in a concentration-dependent manner, and P_3-25 showed more MDA production than other derivatives, suggesting that all these 1,2,4-thiadiazolidine-derivatives are potent inducers of apoptosis as they induce lipid peroxidation. The P_3-25 was more potent than other derivatives.

N-acetyl cysteine inhibits P_3-25 induced ROI generation and cytotoxicity

N-acetyl cysteine (NAC) is known to quench ROI inside the cells. Cells were preincubated with NAC (5 mM) for 1 hr and then treated with P_3-25 (0.1 μM) for 3 hr followed by stimulation with TNF (0.1 nM) for 1 hr. Then ROI generation was assayed by flow cytometry. The results indicated in Figure 9*c*1 that P_3-25 and TNF induced ROI generation alone and showed an additive effect in combination. NAC completely inhibited the ROI generation induced either by P_3-25, TNF or in combination. When cells were pretreated with NAC and then P_3-25, followed by TNF for 72 hr and assayed for cytotoxicity by MTT method, the results showed in Figure 9*c*2 that P_3-25 induced cytotoxicity by 65%, TNF induced by 25% and in combination induced 75%. In NAC pretreated cells, TNF-induced cytotoxicity was not observed, whereas about 50% inhibition was observed in P_3-25- or in combination with TNF-treated cells.

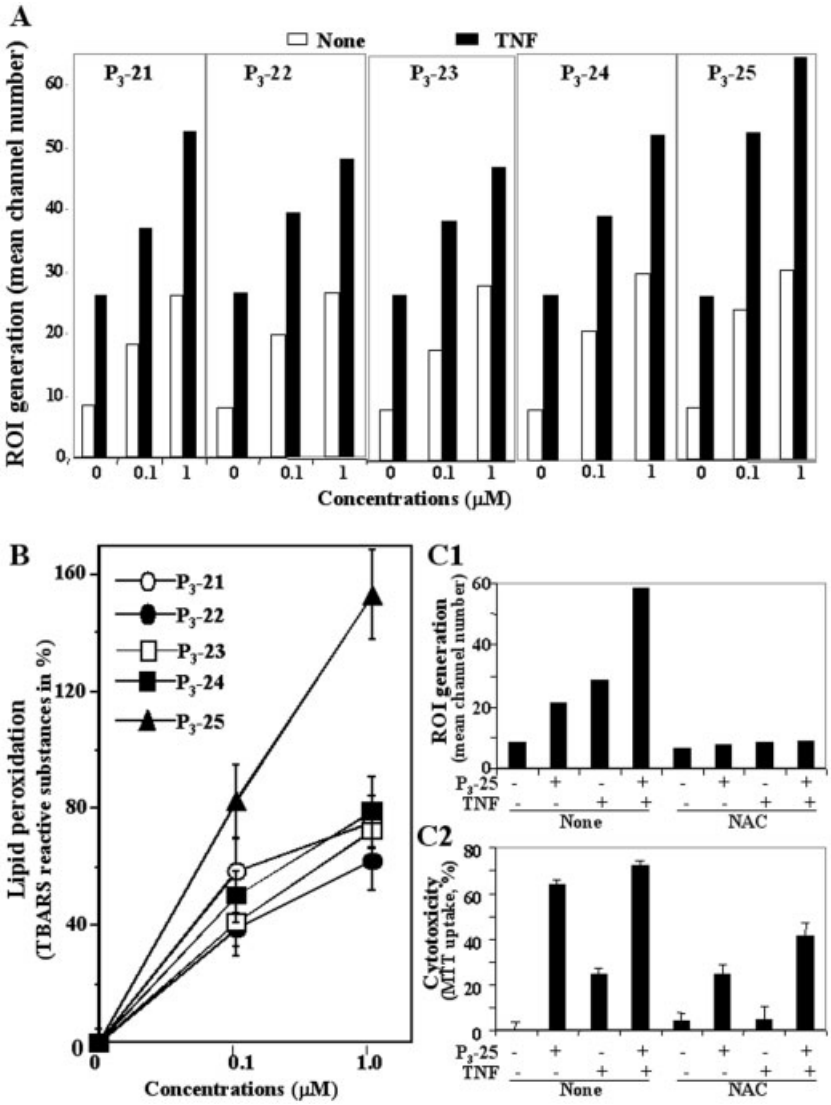

FIGURE 9 – (*a*) Effect of 1,2,4-thiadiazolidine derivatives on TNF-induced ROI generation. U937 cells, treated with different concentrations of 1,2,4-thiadiazolidine derivatives for 3 hr, were exposed to TNF (100 pM) for 1 hr. ROI production was then determined by the flow cytometry. (*b*) Effect of 1,2,4-thiadiazolidine derivatives on lipid peroxidation. U937 cells, treated with different concentration 1,2,4-thiadiazolidine derivatives for 6 hr, were pelleted and extracts were used to assay malondialdehyde as described in Material and Methods. The results shown are representative of 2 independent experiments. (*c*) Effect of NAC on P_3-25 and TNF-induced ROI generation and cytotoxicity. Cells, preincubated with NAC (5 mM) for 1 hr were treated with P_3-25 (0.1 μM) for 3 hr followed by stimulation with TNF (0.1 nM) for 1 hr. Then ROI generation was assayed by flow cytometry. Cells were pre-treated with NAC (5 mM) and then treated with P_3-25 (0.1 μM), followed by TNF (0.1 nM) for 72 hr and assayed for cytotoxicity by MTT method. The results shown are representative of 3 independent experiments.

Discussion

The oxidative debenzylation of 1-substitutedaryl-5-substitutedphenyl-2-S-benzyliso-4-biurets with bromine in chloroform produced the corresponding 1,2,4-thiadiazolidine as the main product. The rate of benzyl group substituted at the sulfur atom in 1-substitutedaryl-5-substitutedphenyl-2-S-benzyliso-4-biurets seems to be an important determinant of the reaction route. The benzyl group could only be eliminated when bromine was the oxidant. In the oxidative debenzylation, the role of the solvent appears to be an important factor. The oxidative debenzylation and cyclisation of related 2-S-benzyliso-4-biurets to the corresponding 1,2,4-thiadiazolidine using chloroform and benzene as a solvent was successfully observed. Using different derivatives of 1,2,4-thiadiazolidine, the anti-inflammatory and anti-tumor activities were determined.

In our report, we demonstrated that different derivatives of 1,2,4-thiadiazolidine can block NF-κB activation, as determined by consensus DNA binding and dependent reporter gene transcription. We found that 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine (P_3-25) is indeed a potent inhibitor of TNF-induced activation of NF-κB and this inhibition is cell line specific. Since TNF is involved in all sorts of inflammatory response and also in tumorogenicity, we used TNF as an inducer of inflammatory response. P_3-25's ability is not only to increase ROI generation, lipid peroxidation and cell death but also potentiates TNF-induced ROI generation and cell death. Modification of different side group(s) of known compound(s) to make it more efficient drug would be viable strategy in the medical science, considering the genesis of in-effective anti-inflammatory and chemotherapeutically resistant drugs. In our study, the dichlorophenyl-derivative (P_3-25) of 1,2,4-thiadiazolidine is much potent than chlorophenyl (P_3-24)- or nonchlorophenyl-derivatives.

Recent evidence indicates that different inflammatory agents may activate NF-κB through the mechanisms that consist of some overlapping and some nonoverlapping steps.[29–31] TNF-induced NF-κB activation was inhibited by P_3-25, suggesting its anti-inflammatory property. How P_3-25 blocks TNF-induced NF-κB activation is at present unclear. Most inhibitors of NF-κB activation mediate their effects through suppression of phosphorylation and degradation of IκBα (e.g., curcumin and silymarin).[27,33,34] Caffeic acid phenethyl ester and resveratrol block NF-κB activation without any effect on IκBα phosphorylation or degradation[35,36] but by inhibiting p65 phosphorylation followed by translocation to nucleus.[37] We showed that P_3-25 does not interfere with the NF-κB consensus DNA binding site (data not shown) but that it did block the TNF-induced translocation of NF-κB to nucleus and reporter gene transcription (Fig. 3). P_3-25 blocks TNF-induced NF-κB activation through inhibition of IκBα degradation (Fig.4B1) and nuclear translocation of p65 subunit of

NF-κB (Fig. 4*c*1,*c*2,*d*). The IκBα degradation is preceded by its phosphorylation and ubiquitination. Phosphorylation of IκBα is regulated by different kinases (IKK-α, IKK-β, IKK-γ, NIK, AKT and MEKK1).[38–41] P_3-25 blocks TNF-induced IKK activation (Fig. 4*b*2), indicating that the IKK may be target for this inhibitor. Therefore, it is possible that some of more upstream kinases may also involve in that P_3-25-mediated inhibition of IKK activation, which needs to be clarified further.

We also detected that NF-κB regulated genes involved in inflammation such as Cox2 are downregulated by different derivatives of 1,2,4-thiadiazolidine as detected by NF-κB-dependent Cox2-linked luciferase reporter gene activation assay (Fig. 6*c*). P_3-25 was shown to be a potent inhibitor of Cox2 activation and it blocked TNF-induced Cox2 protein levels in dose- and time-dependent manner (Fig. 6*a*,*b*), suggesting P_3-25's role for anti-inflammatory activities.

We found that P_3-25 blocked NF-κB activation induced by a wide variety of agents including PMA, LPS, Ceramide, IL-1 and TNF but not by H_2O_2 (Fig. 5*a*). Compared to most other agents, NF-κB activation induced by H_2O_2 follows different kinetic of IκBα degradation and through lipid peroxidation, again suggesting a difference in the signaling pathway.[26,42] Even different tumor cells follows different pathways for H_2O_2-mediated cell signaling.[26] P_3-25 blocked TNF-induced IKK activation and IκBα degradation indicating its mechanism of action unlike H_2O_2.

Though ROI generation causes NF-κB and AP-1 activation, we did not find any NF-κB activation by P_3-25 alone and yet it induced reactive intermediates generation. The amount and long-time exposure with these toxic agents might have deleterious effect to induce cell death as reported previously.[43] The 1,2,4-thiadiazolidine derivatives have shown to induce cyotoxicity and caspase activation (Fig. 7). and P_3-25 was shown to be more potent than other derivatives. Because NF-κB activation has been shown to play an antiapoptotic role,[44] the suppression of NF-κB by P_3-25 may seem to be the cause of potentiation of apoptosis, suggesting NF-κB's role in apoptosis. Our discovery that P_3-25 potentiates TNF-induced ROI generation and lipid peroxidation explains the mechanism by which P_3-25 exerts its effects. NAC inhibits ROI generation mediated by P_3-25 or TNF but about 50% inhibition of cytotoxicity, suggesting that ROI is not the sole agent for P_3-25-mediated cytotoxicity but there may be other mechanisms such as lipid peroxidation, caspases activation *etc*. are involved and this needs to studied further.

Several reports indicate that constitutively or induced NF-κB induces resistance to apoptosis stimulated by a wide variety of agents.[20,45–47] Because P_3-25 has shown cytotoxic effect to tumor cells, it is possible that this toxicity is mediated through the suppression of NF-κB. TNF is well known to induce cytotoxicity to cells. P_3-25 potentiates TNF-induced cytotoxicity as shown by MTT assay possibly by inhibiting NF-κB activation.

Several genes such as cytokines, cyclooxygenase-2, metalloproteases, urinary plasminogen activator and cell surface adhesion molecules are involved in tumor promotion and are also regulated by NF-κB.[48–51] Since P_3-25 blocked NF-κB-dependent reporter gene expression, it may play a critical role in carcinogenesis and inflammation exhibiting anti-carcinogenic and anti-inflammatory effects. Inhibiting NF-κB by adenoviral IκBα or proteosome inhibitors are currently being tested to overcome chemotherapy-induced resistance.[45] Therefore, NF-κB suppressive ability of P_3-25 could be exploited by combination with chemotherapy. Owing to its ability to suppress Cox2 through NF-κB, aspirin is beneficial for preventing colon cancer.[52] This suggests that P_3-25 may also prove to be beneficial for colon cancer. P_3-25's ability to suppress TNF-induced NF-κB and other cellular responses may provide the molecular basis for the anticarcinogenic properties of P_3-25. Our results suggest that P_3-25 may also have applications for various other diseases including inflammation and arthritis, where NF-κB activation has been shown to mediate pathogenesis. These possibilities require further investigation in detail.

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