Biochemical Pharmacology 78 (2009) 495–503

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Novel role of thiadiazolidine derivatives in inducing cell death through Myc-Max, Akt, FKHR, and FasL pathway

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ARTICLE INFO

Article history:
Received 20 March 2009
Accepted 30 April 2009

Keywords:
1,2,4-Thiadiazolidine
Cell signaling
Apoptosis
FasL
Myc
Cell cycle

ABSTRACT

The 1,2,4-thiadiazolidine derivatives show anti-fungal and anti-inflammatory activities. We previously reported that these derivatives inhibit nuclear factor-kappaB (NF-κB), a transcription factor that induces tumorigenesis through activation of several genes. We have aimed to elucidate the mechanism of apoptosis mediated by these derivatives. In this study we provide evidence that dichlorophenyl form of thiadiazolidine (designated as P_3-25) is a potential inducer of cell death by arresting cell cycle at G1 phase and decreases the amounts of cyclin D1 and cyclin E without interfering p16 and p27. It decreased c-Myc level and thereby inhibited DNA binding ability of Myc-Max complex. P_3-25 dephosphorylated Rb and Akt facilitating nuclear translocation of FKHR that then expressed gene FasL. Activated FasL inhibited cell proliferation and induced cell death. Our results suggest that P_3-25 derivative exerts anti-tumor activities by decreasing Myc-mediated response and increasing FasL expression, which may help in designing drugs for tumor therapy.

1. Introduction

The thiazolidones and thiadiazoles have drawn considerable attention for their anti-bacterial [1], anti-fungal [1], and anti-inflammatory [2] activities. Considering anti-fungal activity of some of nitrogen and sulfur containing 1,2,4-thiadiazolidines and 3-oxo-1,2,4-thiadiazolidines [3], prompted us to synthesize newer 1,2,4-thiadiazolidines by employing oxidative debenzylation technique [4] and study their biological activities. Previously, we reported that these derivatives inhibited TNF-induced NF-κB activation through IKK inactivation [5]. In constitutive NF-κB expressing cells these thiadiazolidine derivatives especially dichlorophenyl form (designated as P_3-25) inhibited upstream kinases such as protein kinase A (PKA) and casein kinase 2 (CK2) and downregulated NF-κB-dependent gene expression [6]. Controlled cell cycle arrest seems to be the key mechanism to kill tumor cells induced by chemotherapy, radiation, and cytokines such as TNF [7,8].

In association with Max, c-Myc induces several genes involved in the control of cell proliferation such as cyclin D1 [9], cyclin D2 [10], cdc25a [11], p21Cip1 [12], p15Ink4b [13], and cdk4 [14]. Retinoblastoma (Rb) protein binds with elongation transcription factor 2 (E2F) and represses cell cycle. In the growth phase, Rb has been phosphorylated by upstream Cdks and removed from Rb-E2F complex and thereby mediates E2F-dependent gene transcription that promotes cell proliferation i.e. transition from G1 to S-phase. Cyclin D1 mediates transactivation of several genes partnering with CDK4/6 for the G1/S cell cycle growth [14]. Factors that exert cell growth-inhibitory effects are p21 (expressed by p53), p15 and p27 (stimulated by transforming growth factor) and the senescence protein p16. These proteins have been shown to bind with cyclin/CDK complexes, thereby preventing phosphorylation of Rb and thus inhibiting cell proliferation [15].

By phosphorylating several downstream targets which include effectors such as Forkhead transcription factors (FKHR), a serine threonine kinase, Akt/PKB inhibits apoptosis [16,17]. Phosphorylated Akt that contributes in tumorigenesis in different tumors [18,19] is known to arrest FKHR in the cytoplasm, and its dephosphorylation leads to nuclear translocation of FKHR [17]. Cdc25A, a phosphatase frequently present in all forms of tumors and expressed by c-Myc, promotes cell

Abbreviations: Bim, Bcl-2 interacting mediator of cell death; FBS, fetal bovine serum; P_3-25, 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-Oxo-1,2,4-thiadiazolidine; Rb, retinoblastoma; p-Rb, phospho-Rb; FKHR, forkhead transcription factor.

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doi:10.1016/j.bcp.2009.04.032

progression by activating G1 CDKs. Cdc25A is physically associated with apoptosis-signal-regulating kinase 1 (Ask1) and activates mitogen activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) [20,21].

Cells are acquiring resistance to some of the effective agents/drugs by yet unknown mechanisms. Understanding the cell signaling cascade that interacts with such novel molecule(s) that are potential drug(s) with low side effects would be viable strategy for combination therapy. In this report we are providing data that some 5-substitutedarylimino-2-N-substitutedphenyl-3-oxo-1,2,4-thiadiazolidine derivatives inhibit cell cycle by arresting at G1/S phase. Synthesis of these compounds is less laborious thereby this should be cheaper if it is used as therapeutic medicine. These compounds did not show any cytolysis effect even 48 h of incubation. Details molecular mechanism of action mediated by these compounds is required to address to resistance mechanism if generated at all in the cells. Among these derivatives the 5-(4-methoxyarylimino)2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine, designated as P_3-25, is shown to be more potent in inducing cell death. P_3-25 dephosphorylates Akt and thereby inducing nuclear translocation of FKHR. FKHR in turn induces FasL expression, which induce cell death. It decreases phospho-retinoblastoma level and suppresses c-Myc-mediated transcription, which further induces cell death. This observation may be helpful to design P_3-25 as a novel anti-inflammatory and/or anti-tumor drug.

2. Materials and methods

2.1. Materials

Propidium iodide (PI), glycine, and anti-tubulin antibody were obtained from Sigma–Aldrich Chemicals (St. Louis, MO). Penicillin, streptomycin, RPMI-1640 medium, and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY, USA). Antibodies against cyclin B, cyclin D1, cyclin E, p21, Cdc25A, c-Myc, Bim, Rb, Akt, FKHR, CRM1, FasL, and goat-anti-rabbit IgG conjugated with HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-Akt and -Rb were purchased from Cell Signaling Technologies (Danvers, MA, USA). The annexin V-PE and goat anti-rabbit IgG-fluorescein or -Alexa Fluor were obtained from Molecular Probe (Eugene, OR, USA). The 5-(4-Methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidines were synthesized from 1-(4-methoxyaryl) thiocarbamide as described previously [4,5].

2.2. Cell culture

The human U-937 (histiocytic lymphoma) and HeLa (Human epithelial carcinoma) cells were obtained from American Type Culture Collection (Manassas, VA, USA). The cell line was cultured in RPMI-1640 medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were free from mycoplasma, as detected by Gen-Probe mycoplasma detection kit (Fisher Scientific, PA, USA) at 37 °C in 5% CO_2.

2.3. Cell proliferation assay (^{3}H-thymidine incorporation)

The viable and proliferating cell number was detected by ^{3}H-Thymidine incorporation assay. U-937 cells (10^4 cells/well of 96-well plate) were incubated with test sample in a final volume of 0.2 ml for 48 h at 37 °C. Cell proliferation was measured by thymidine incorporation by adding 50 μl of ^{3}H-thymidine (0.5 μCi/well diluted in Hank's buffered salt solution) last 18 h. Cells were harvested and washed, and thymidine incorporation was measured in a beta counter (Packard).

2.4. Cell cycle and apoptosis analysis

The collected cells (control and treated) were fixed with 70% ethanol and were suspended in DNA-staining buffer (50 μg/ml propidium iodide, 1% Triton X-100, 0.1 M phosphate-buffered saline) for 30 min at 4 °C. Cells were then subjected to flow cytometry analysis for cell cycles and apoptosis [22]. The apoptotic cells were detected by Annexin V-PE apoptosis detection kit (Molecular Probe, Eugene, OR). After different treatments, cells were washed with PBS and suspended in 1× binding buffer and incubated with Annexin V-PE and 7 AAD at room temperature for 15 min. Then cells were analyzed in FACS Scan.

2.5. Western blot analysis

Cyclins, Phospho-Rb, Rb, phospho-Akt, Akt, FasL, FKHR, p21, c-Myc, Bim, Cdc25A, CRM1, proteinase 3, actin and tubulin were detected by Western blot technique using specific antibodies followed by detection by chemiluminescence (Amersham Pharmacia Biotech, NJ, USA).

2.6. Immunocytochemistry

The levels of different phosphorylated proteins were examined by the immunocytochemical method as described [22]. Briefly, HeLa cells, plated on a poly-L-lysine-coated glass slide were air-dried, fixed with 3% formaldehyde, and permeabilized with 0.1% of Triton X-100. Slides were incubated with anti-p-AKT, -c-Myc, -p16, or p27 antibody for 6 h followed by incubation with anti-rabbit IgG-FITC or -Alexa Fluor for 1 h. Slides were mounted with mounting medium with DAPI and analyzed under a fluorescence microscope.

2.7. Myc-Max DNA binding activity assay

To determine Myc-Max activation, EMSA were conducted essentially as described [23]. Briefly, cells, after different treatments were used to prepare cytoplasmic and nuclear extracts. Nuclear extract proteins (8 μg) were incubated with ^{32}P end-labeled double-stranded Myc-Max oligonucleotide having sequence of 5′-GGA AGC AGA CCA CGT GGT CTG CTT CC-3′ for 30 min at 37 °C, and the DNA–protein complex was separated from free oligonucleotide on 6.6% native PAGE. The specificity of binding was examined by competition with unlabeled oligonucleotide. Visualization of radioactive bands was done in a PhosphorImager (Fuji, Japan).

2.8. FasL-dependent reporter gene transcription

The level of FasL-dependent luciferase reporter gene expression was carried out as described previously [24]. U-937 cells were transiently transfected with lipofectamine 2000 reagent containing FasL-luciferase (0.5 μg) and GFP (0.5 μg) constructs. After 3 h of transfection, cells were washed and cultured for 12 h. GFP positive cells were counted (27–30% for different combinations). Cells were treated with 100 nM P_3-25 for different times. The cell pellets were collected and extracted with 1× lysis buffer (part of luciferase assay kit from Promega). The lysate was incubated in ice for 10 min and then froze in liquid nitrogen quickly thawed at 37 °C. It was then centrifuged at maximum speed for 5 min at 4 °C. The supernatant was removed carefully and allowed to come to room temperature. 100 μl of the extract was incubated with 25 μl firefly luciferin substrate from Promega (Madison, WI, USA). Emission of light was read from luminometer. 5 μl of the sample was used and the amount of protein present was estimated using Bradford assay and values were calculated as RLU/mg protein.

2.9. Cytotoxicity assay

2.9.1. MTT assay

The cytotoxicity was assayed by the MTT dye reduction [25]. Briefly (10^4 cells/well of 96-well plate), were incubated with test sample in a final volume of 0.1 ml for 72 h at 37 °C. Thereafter, 25 μl of MTT solution (5 mg/ml in PBS) was added to each well. After a 2-h incubation at 37 °C, 0.1 ml of the extraction buffer (20% SDS, 50% dimethylformamide) was added. After an overnight incubation at 37 °C, the absorbance was read at 570 nm using a 96-well multiscanner autoreader (Biorad), with the extraction buffer as a blank.

3. Results

Different 1,2,4-thiadiazolidine derivatives (P_3-21 to P_3-25) were chemically synthesized in the laboratory and their structures of compounds confirmed by element analyses, IR, ^{1}H NMR, ^{13}C NMR and mass spectra [5] (Fig. 1). All these derivatives were dissolved in DMSO at 10 mM concentration. Further dilution was carried out in the medium. In this study, we used U-937 and HeLa cells which are well characterized in our laboratory for different cellular activity. The concentrations and times used for different derivatives for this study had no effect in cytolysis as detected by lactate dehydrogenase (LDH) assay [Culture supernatant from 100 nM P_3-25 treated HuT-78 cells for 0, 24, and 48 h when incubated with substrate solution (0.23 M sodium pyruvate and 5 mM NADH in 0.1 M phosphate buffer, pH 7.5) showed 1.05 ± 0.04, 1.02 ± 0.07, and 0.989 ± 0.062 absorbencies, respectively at 340 nm].

3.1. Thiadiazolidine-derivatives induce cell death in U-937 cells

U-937 cells were treated with varying concentrations of thiadiazolidine derivatives (P_3-21, P_3-22, P_3-23, P_3-24 or P_3-25) for 72 h and cell viability was measured by MTT assay. Cell viability was decreased by all these derivatives as shown by the decrease in absorbance in concentration-dependent manner (Fig. 2A). Cell death was observed at 47, 48, 54, 60, and 75% by P_3-21, P_3-22, P_3-23, P_3-24 and P_3-25 respectively ($p < 0.005$). The IC50 for P_3-21, P_3-22, P_3-23, P_3-24 or P_3-25 are 124, 120, 45, 22, and 7.8 nM, respectively. U-937 cells treated with P_3-21, P_3-22, P_3-23, P_3-24 or P_3-25 (100 nM each for 48 h) showed 18%, 21%, 29%, 36%, or 58% cell death analyzed by FACS using annexin V-PE staining, respectively (Fig. 2C). P_3-25 inhibited cell proliferation as monitored by ^{3}H-thymidine incorporation in a dose-dependent manner (Fig. 2B). These data suggest that thiadiazolidine-derivatives potently induce apoptosis.

3.2. Thiadiazolidine-derivatives arrest cell cycle at G1/S phase in U-937 cells

U-937 cells were treated with different thiadiazolidine-derivatives (100 nM) and curcumin (1 mM) for different times, Most of the cells were found in the G1 phase by those derivatives and at 48 h of treatments, cell population increased at G0 phase as indicated in bar diagram in percentage (Fig. 3). Curcumin showed similar response. These results suggest that thiadiazolidine-derivatives arrest cells at G1/S phase like curcumin.

3.3. P_3-25-mediated cell death is not cell type specific

As biological activities differ in different cell types, 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 epithelial and lymphoid cells. The cell death mediated by P_3-25 was carried out in U-937 cells, a histiocytic lymphoma. We found that P_3-25 induced cell death in human macrophage (THP-1) and human lung carcinoma (A549), epithelial cell line (HeLa), and breast carcinoma (MCF-7) cells within 46% to 54% ($p < 0.01$) (Fig. 4). These results suggest that the cell death effect of P_3-25 is not restricted to specific cells.

3.4. P_3-25 decreases amounts of cyclin D1, cyclin E, cyclin B, or phospho-Rb, but not Bim, Rb, p16, or p27

The high basal amounts of cyclin D1, cyclin E, cyclin B, and phospho-Rb decreased with the treatment of P_3-25 in a time-dependent manner as assayed from U-937 cell extracts by Western

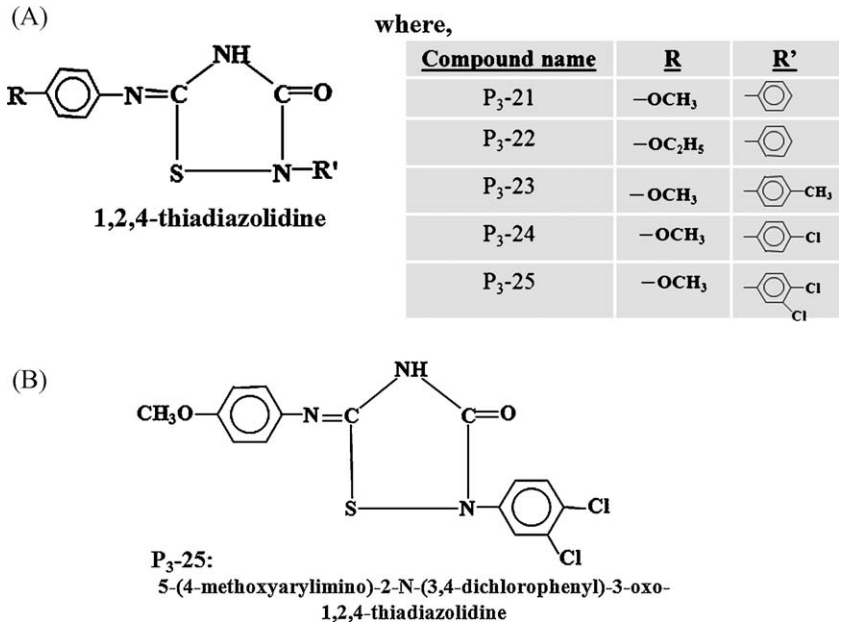

Fig. 1. Structure of 1,2,4-thiadiazolidine derivatives (P_3-21 to P_3-25) where R and R′ are indicated in the Table (A). The structure of P_3-25 is indicated (B).

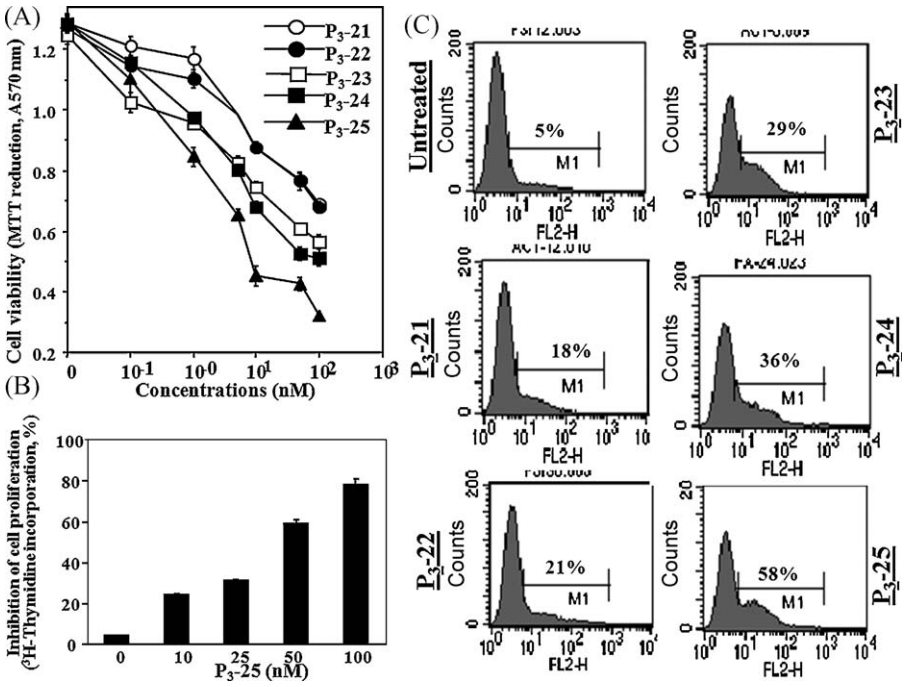

Fig. 2. Effect of thiadiazolidine derivatives on cell death. U-937 cells (5×10^3/well of 96-well plate in 100 μl) were treated with varying concentrations of thiadiazolidine derivatives (P_3-21, P_3-22, P_3-23, P_3-24 or P_3-25) in triplicate for 72 h. Cell viability was detected by MTT assay and mean absorbance ±SD was indicated in the figure (A). U-937 cells (5×10^3/well of 96-well plate in 100 μl) were treated with varying concentrations of P_3-25 for 48 h and ^{3}H-thymidine was added in each well for last 18 h. Radioactivity was counted in Beta-Counter and decrease in the counts is indicated as inhibition of cell proliferation in percentage (B). U-937 cells were treated with 100 nM each of P_3-21, P_3-22, P_3-23, P_3-24, or P_3-25 for 48 h. Cell death detected by Annexin V-PE and analyzed in FACS (C).

blot (Fig. 5A and B). The amount of Bim increased at 36 and 48 h of P_3-25 treatment. The amount of Rb remained unchanged for these treatments. The concentrations of p16 or p27 remained unchanged in 100 nM of P_3-25-treated HeLa cells at 24 h or 36 h of treatments as shown by immunofluorescence study (Fig. 5C). These results suggest that P_3-25 treatment decreases amounts of cyclins and phospho-Rb and increases amount of Bim without altering the amounts of Rb, p16, or p27.

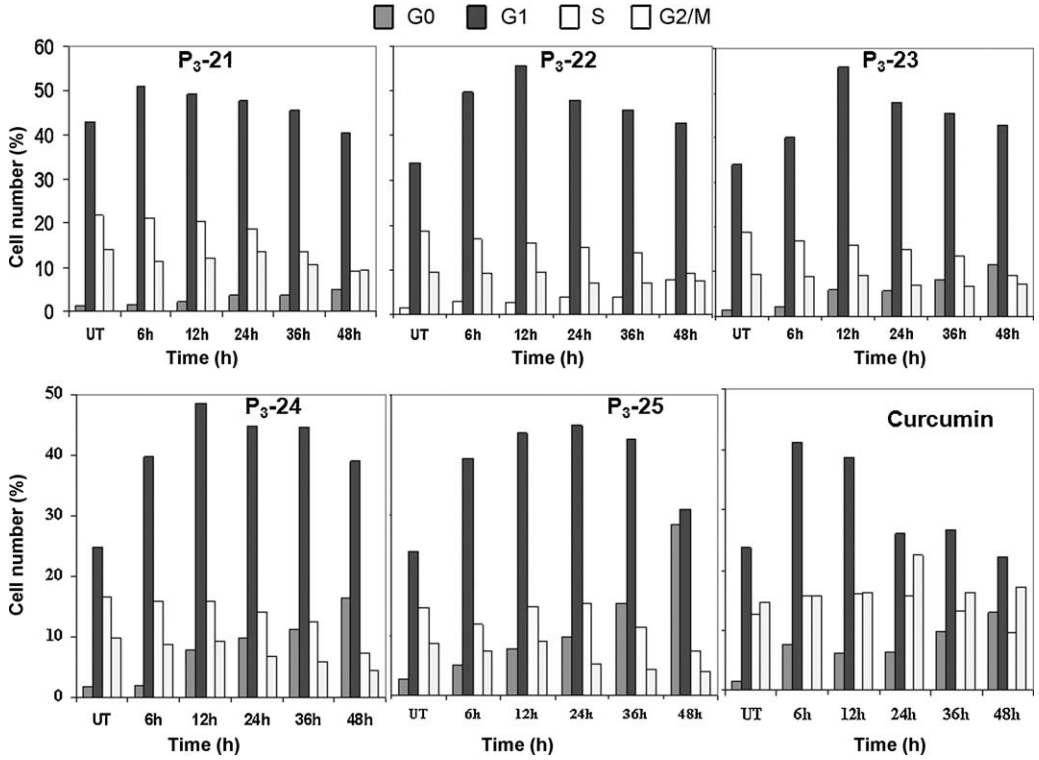

Fig. 3. Effect of thiadiazolidine derivatives on cell cycle. U-937 cells were treated with 100 nM each of P_3-21, P_3-22, P_3-23, P_3-24, or P_3-25 for different times. Cells were stained with propidium iodine, and the number of apoptotic cells was counted with a FACSCalibur flow cytometer. Each panel shows cell cycle at 48 h in percentage for different times of P_3-25 treatment of various stages of the cell cycle [G0 (apoptosis), G_1, S, and G_2/M]. Results presented in figures are representation of one out of three independent experiments.

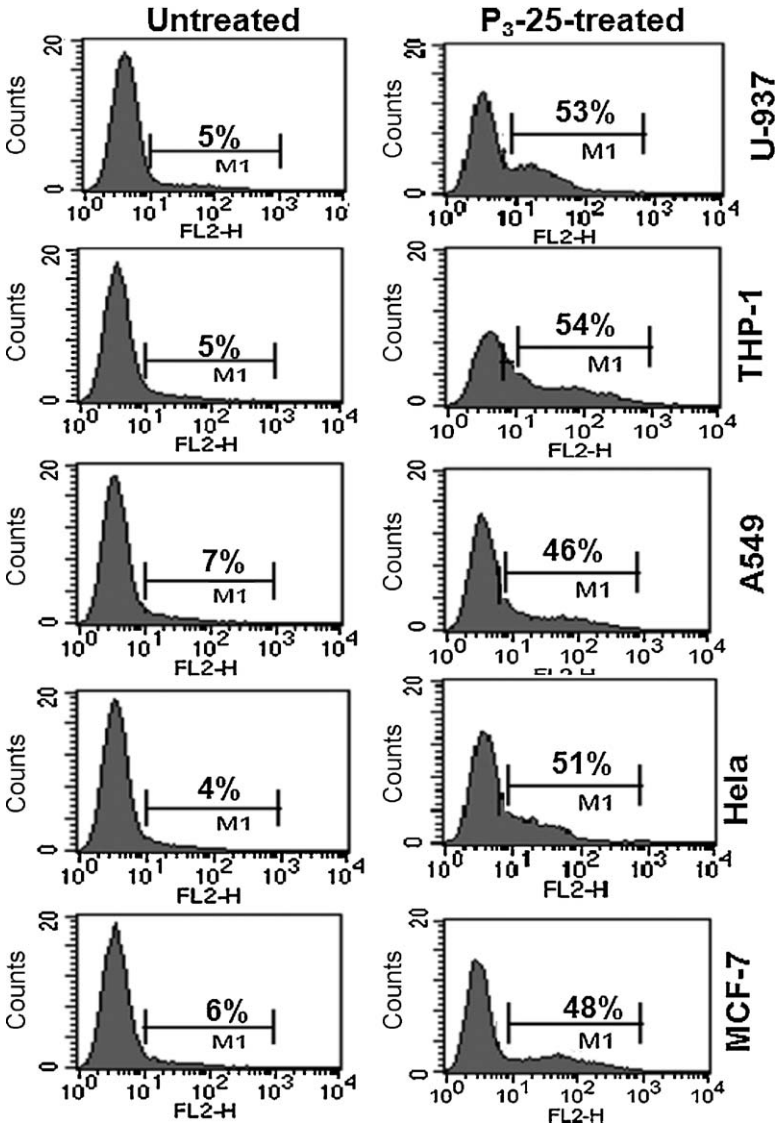

Fig. 4. Effect of P_3-25 on cell death in different cell types. U-937, THP-1, A549, HeLa, and MCF-7 cells were treated with 100 nM of P_3-25 for 48 h. Cell death detected by Annexin V-PE and analyzed in FACS.

3.5. P_3-25 decreases the amount of c-Myc and DNA binding activity of Myc-Max complex

DNA binding activity of the Myc-Max was decreased in the nuclear extracts obtained from P_3-25-treated cells, as measured by gel shift assay (Fig. 6A). Unlabeled Myc-Max oligonucleotide (50-fold) completely abolished or partially shifted the radioactive band by anti-c-Myc antibody (Fig. 6B) suggesting specificity of Myc-Max complex. The concentration of c-Myc, as shown by immunofluorescence (Fig. 6C) and Western blot (Fig. 6D, upper panel) decreased in P_3-25-treated cells. These data suggest that P_3-25 interferes c-Myc activities in combination with Max. Also the expression of Myc-Max-dependent genes, p21 and Cdc25A decreased with increasing time of P_3-25 treatment (Fig. 6D, middle and lower panels).

3.6. P_3-25 decreases the amount of phospho-Akt and enhances nuclear translocation of FKHR

The amount of phospho-Akt decreased in P_3-25-treated cells, as detected by immunofluorescence, in HeLa cells (Fig. 7A). The amount of phopspho-Akt decreased with increasing time of P_3-25 treatment in U-937 cells, as assayed by Western blot (Fig. 7B). The amount of Akt remained same in all the time points. The amount of FKHR gradually decreased in the cytoplasmic extracts with increasing time of P_3-25 (100 nM)-treatment, which correlated with gradual increase in the amounts of FKHR in nuclear extracts (Fig. 7C). These results suggest that P_3-25 enhances nuclear translocation of FKHR.

3.7. P_3-25 induces FasL expression

The amount of FasL increased in P_3-25-treated cells as observed in culture supernatant and cell pellet (Fig. 7D). Anti-FasL antibody protected P_3-25-induced cell death, as determined by MTT assay (Fig. 7E). FasL-dependent luciferase gene expression, as analyzed by luciferase activity, increased in P_3-25-treated cells in a time-dependent manner in *FasL-luciferase* construct transfected U-937 cells (Fig. 7F), further indicated the P_3-25-mediated expression of FasL.

3.8. P_3-25 induces caspase 3 and 8 activation

As P_3-25 induces FasL expression, the activities of caspase 3 and 8 were also assayed. The amounts of active casepase 3 or 8 (cleaved

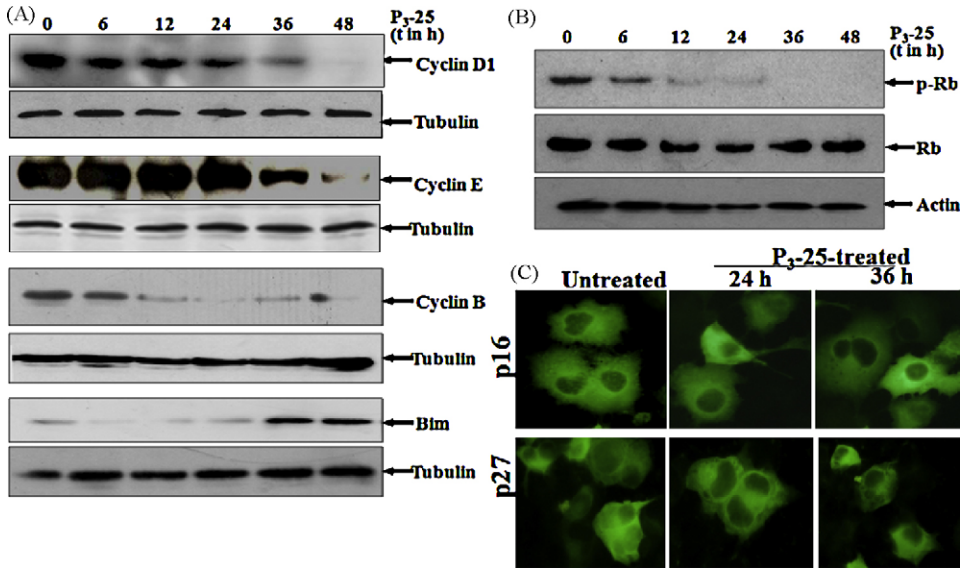

Fig. 5. Effect of P_3-25 on amounts of cyclin D1, cyclin E, cyclin B, Bim, phospho-Rb, p16, and p27. U-937 cells were treated with 100 nM of P_3-25 for different times. The aomunts of cyclin D1, E, and B and Bim were detected from whole cell extracts by Western blot; same blots were reprobed for tubulin (A). The level of phospho-Rb was detected from whole cell extract proteins from same treatments. The same blot was re-probed for Rb and actin by Western blot (B). HeLa cells were treated with 100 nM of P_3-25 for 24 h and 36 h and then cells were fixed, incubated with anti-p16 or -p27 antibodies and immunoflourescence was detected by FITC-tagged secondary antibody in Fluorescent microscope (C).

from procaspase 3 or 8) proteins were observed in P_3-25-treated U-937 cells at 24 h of treatment (Fig. 8A and B). The active caspases bands were observed in doxorubicin-treated cells suggesting the role of P_3-25 as an inducer of cell death. The activities of caspase 3 and 8 increased in P_3-25-treated cells after 12 h of incubation (Fig. 8C), which is correlated with the expression with FasL expression.

3.9. Phosphatase or caspase inhibitor partially protects P_3-25-mediated cell death

Pre-treatment of okadaic acid, a phosphatase inhibitor, z-VAD-fmk, a caspase inhibitor, and anti-FasL antibody led to considerable amount of protection of P_3-25-mediated cell death as detected by FACS using annexin-V-PE (Fig. 9A). Okadaic acid protected P_3-25-

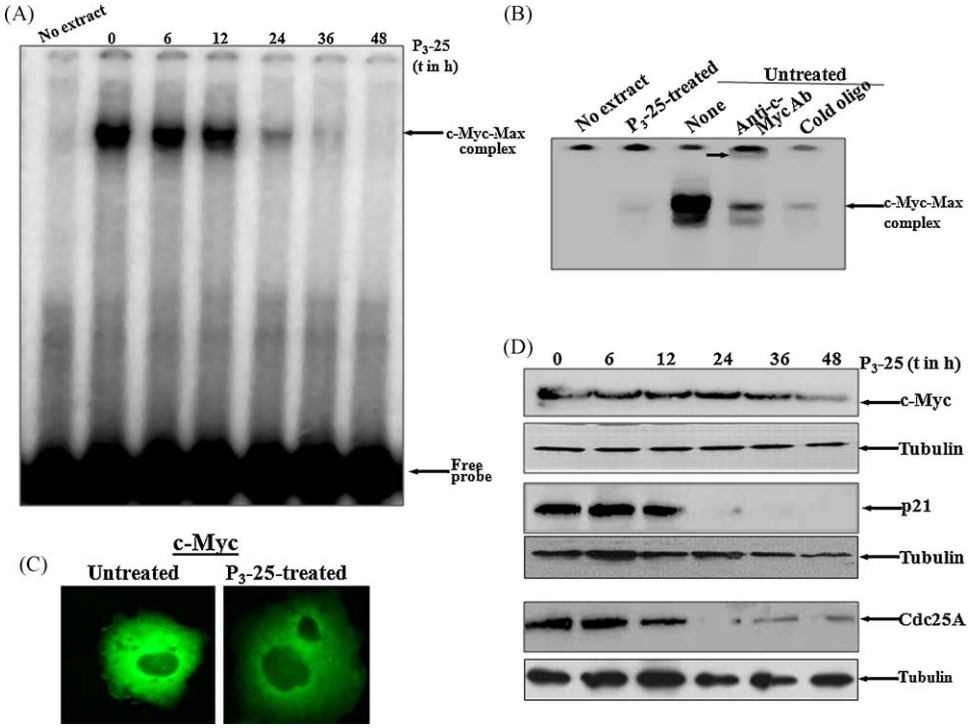

Fig. 6. Effect of P_3-25 on c-Myc-Max DNA binding activity. U-937 cells were treated with 100 nM of P_3-25 for different times, then DNA binding activity c-Myc-Max complex assayed from nuclear extracts by gel retardation (A). Nuclear extracts were incubated for 15 min with anti-c-Myc antibody or unlabeled c-Myc-Max complex oligo, and then assayed for c-Myc-Max complex activation by gel retardation (B). The level of c-Myc was detected from untreated and P_3-25-treated (100 nM for 12 h) HeLa cells by immunofluorescence (C). The amounts of c-Myc, p21, and Cdc25A were measured from P_3-25 (100 nM)-treated cells for different times by Western blot (D).

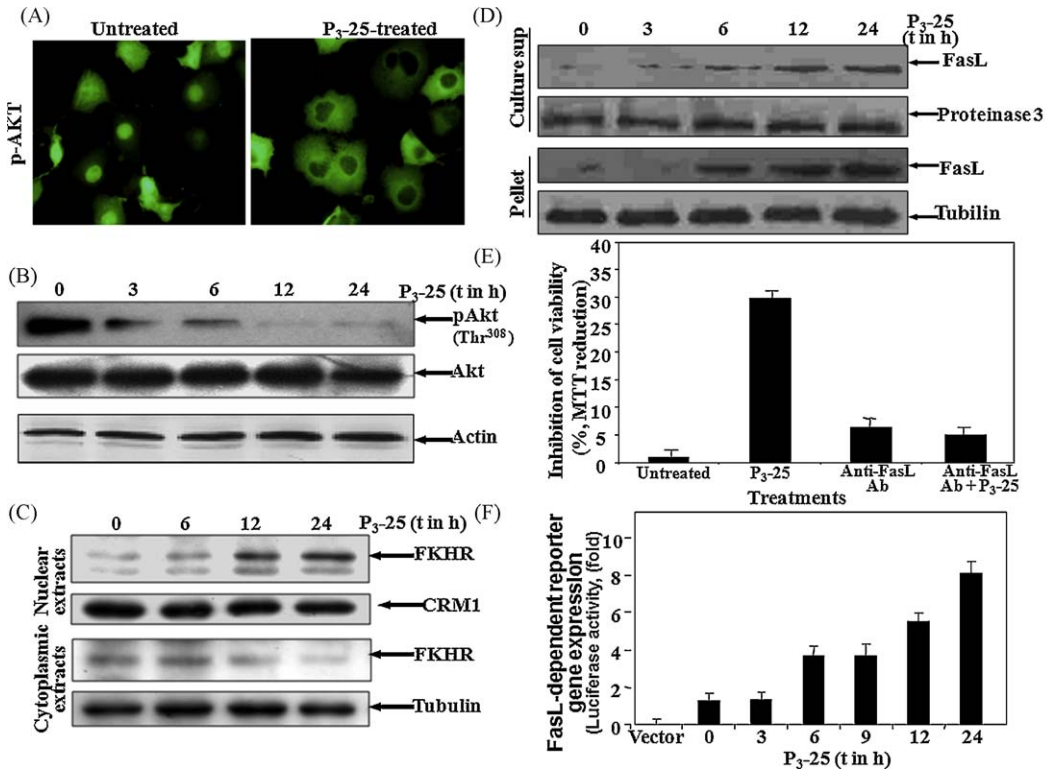

Fig. 7. Effect of P_3-25 on amounts of phospho-Akt, nuclear translocation of FKHR, and expression of FasL. HeLa cells were treated with P_3-25 for 12 h and then cells were fixed and incubated with anti-phospho-Akt antibody and fluorescence was detected using Alexa-Fluor-tagged secondary antibody by Immunofluorescence microscope (A). The level of phospho-Akt was detected from P_3-25-treated U-937 cells for different times by Western blot (B). The same blot was probed for Akt and actin. Nuclear and cytoplasmic extracts from P_3-25 (100 nM)-treated cells for different times were used to detect FKHR by Western blot and blots were reprobed for CRM1 and tubulin respectively (C). U-937 cells were treated with 100 nM P_3-25 for different times. FasL was detected from culture supernatant (10 times concentrated) and whole cell extracts by Western blot (D) and the blots were reprobed for proteinase 3 and tubulin respectively. U-937 cells were incubated with anti-FasL antibody (1 μg/ml) for 2 h in triplicate and then treated with P_3-25 (100 nM) for 24 h. Cell viability was assayed using MTT dye. Results represented as inhibition of cell viability in percentage, which is calculated from mean absorbance ±SD of triplicate samples (E). U-937 cells, transfected with FasL-luciferase construct were treated with P_3-25 for different times. Luciferase activity was assayed from whole cell extracts (F).

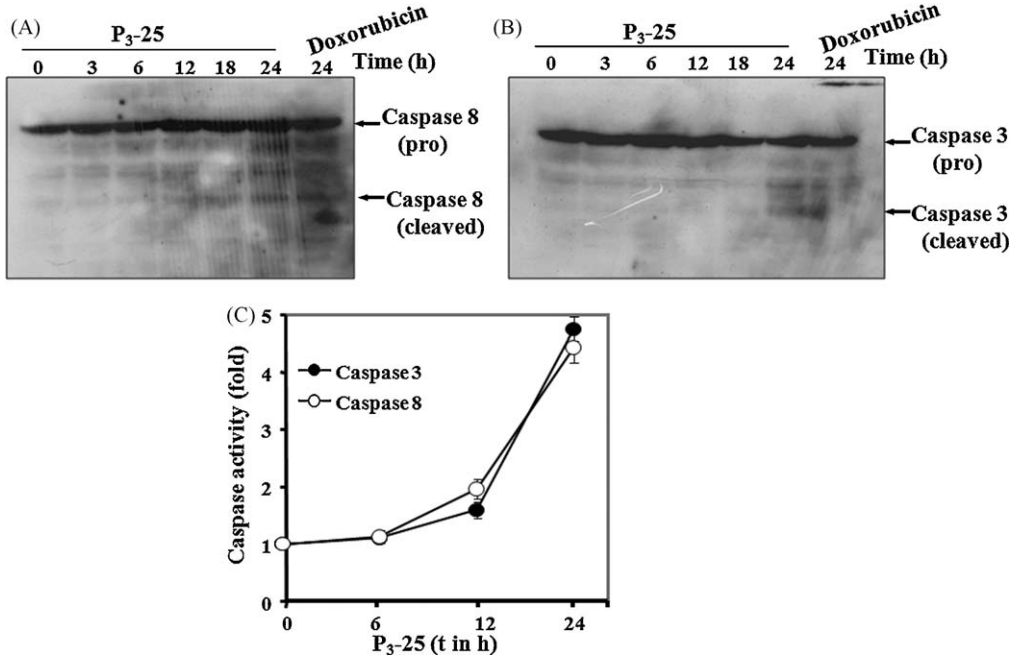

Fig. 8. Effect of P_3-25 on caspase 3 and 8 activation. U-937 cells were treated with 100 nM P_3-25 for different times or 1 μM doxorubicin for 24 h. Whole cell extracts were prepared and 200 μg of proteins were analyzed in 12% SDS-PAGE and caspase 3 and 8 were detected by Western blot (A & B). U-937 cells were treated with 100 nM P_3-25 for different times and caspase 3 and 8 activities were measured and indicated as fold of activation considering untreated cells as 1 fold (C).

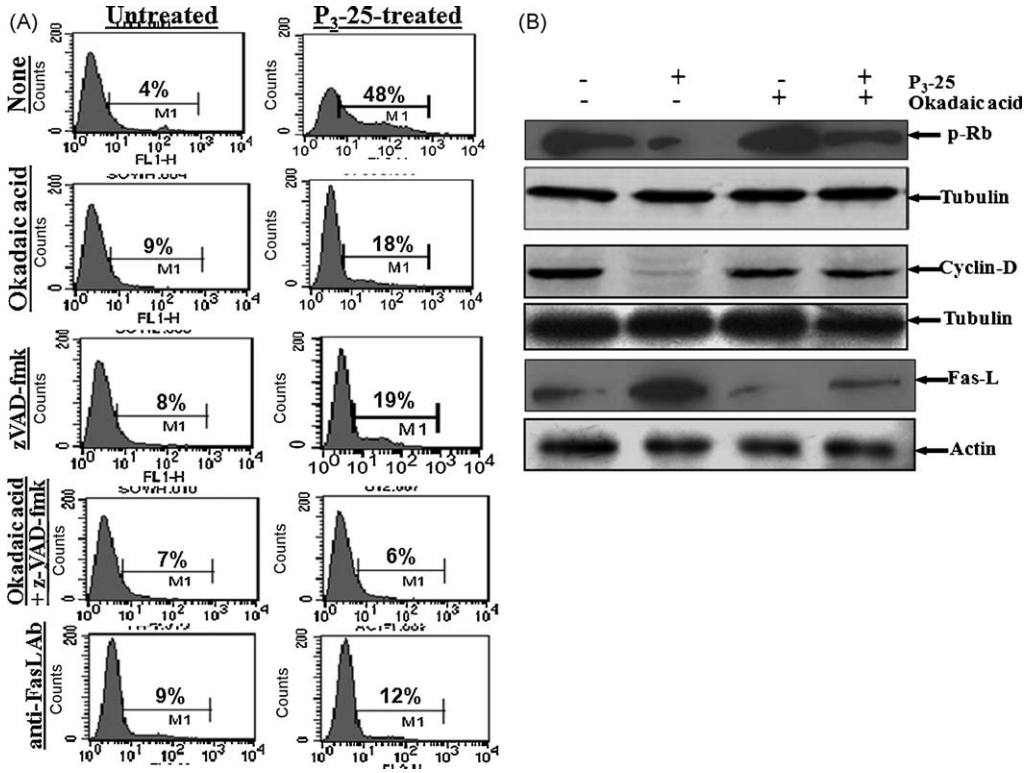

Fig. 9. Effect of okadaic acid, z-VAD-fmk, and anti-FasL antibody on P_3-25-mediated cell death and amounts of p-Rb, cyclin D1 and Fas-L. U-937 cells were pretreated with anti-FasL antibody (1 μg/ml), okadaic acid, or z-VAD (50 nM each) for 4 h and then treated with P_3-25 (100 nM) for 24 h. Cell death detected by annexin V-PE and analyzed in FACS (A). U-937 cells, pretreated with 50 nM okadaic acid for 4 h were treated with P_3-25 (100 nM) for 24 h. Cell extracts were detected for phospho-Rb, cyclin D1 and FasL (B). Blots were reprobed for tubulin.

mediated decrease of phospho-Rb and cyclin D1 and increase of FasL partially (Fig. 9B). These results suggest that phosphatase inhibitor partially protected P_3-25-mediated cell death.

4. Discussion

Understanding the mechanism of action of any compound for its biological activities would be useful for long-term efficacy and also attainment of resistance. In the present report, we demonstrated that thiadiazolidine-derivatives, synthesized from thiourea, are potent inducers of cell death. The dichlorophenyl form of 1,2,4-thiadiazolidine (P_3-25) is most potent among them. Like curcumin, a natural product from turmeric has shown to arrest G1/S cell cycle arrest, the thiadiazolidine-derivatives arrest cell cycle at same phase for a long time suggesting their efficacy as potent inhibitors. P_3-25 was found to inhibit transcription of nuclear transcription factor kappaB (NF-κB)-dependent genes by decreasing p65 phosphorylation through inhibition of PKA and casein kinase 2 in constitutive NF-κB-expressing and doxorubicin-resistant breast cancer cells [6]. c-Myc in combination of Max binds with specific promoter site and induces several genes such as p21Cip1 and Cdc25A [11,12]. Cdc25A enhances cyclin D and cyclin E with its kinase CDK4/6 and CDK2 respectively and these involve in cell cycle progression at G1/S. Cyclin E stabilizes cells at G2/M phase. As amount of cyclin B has shown to be decreased with increasing time of P_3-25 treatment, it is obvious that P_3-25 has no role in G2/M phase arrest. P_3-25 not only decreases DNA binding ability of Myc-Max and thereby amount of Cdc25A, but also the amounts of cyclins. How the amounts of cyclins are decreasing in the cells with P_3-25 treatment needs to be study further. Involvement of different proteases or proteasome and inhibition of different transcription factors might be work out. Tumor suppressers such as p16 or p27 are not affected by P_3-25 treatment and thus suggesting that p53 may be not involved in P_3-25-mediated cell cycle arrest.

Phosphorylation of different signaling molecules is the key factor for cell proliferation and high level of such phospho-proteins are often noticed for tumorigenic response. P_3-25 decreases the amount of phospho-Rb and -Akt. Dephosphorylation of Akt facilitates nuclear translocation of FKHR, which in turn, induces expression of FasL [22]. P_3-25 treatment decreased the amount of phospho-Akt thereby facilitated translocation of FKHR and expression of FasL, which induces cell death. The cdc25A, cdc25B, and cdc25C are a family of human phosphatases that activate the cyclin-dependent kinases at different points of the cell cycle. Overexpression of Cdc25A, a Myc-dependent phosphatase leads to higher proliferative activity of the tumors [26]. P_3-25 treatment decreases the amount of Cdc25A, which might have role in cell death. Okadaic acid, phosphatase inhibitor, partially inhibited P_3-25-mediated decrease in the amount cyclin D, increase the expression of FasL, and induce cell death. These events might lead to understand the increase in the activities of phosphatases by P_3-25 treatment. To induce cell death, the cell-extrinsic pathway is initiated by FasL and cell-intrinsic pathway involves Bim (Bcl-2 interacting mediator of cell death) [27]. P_3-25 not only expressed FasL, but also Bim. Both pathways converge to induce caspases. How P_3-25 induces Bim needs to be studied. The zVAD-fmk, a pan caspase inhibitor partially inhibited P_3-25-induced cell death. Thus, P_3-25 mediates cell death by inducing caspases, inhibiting phosphatases, and regulating phospho-proteins.

P_3-25 has shown to inhibit p65 phosphorylation by blocking upstream kinases—casein kinase 2 and protein kinase A in NF-κB-expressing cells [6,28]. It inhibited TNF-induced cell signaling by inhibiting IκBα kinase (5) possibly by interfering TRAF2 (unpublished observation). Our results suggest that dichlorophenyl derivative of 1,2,4-thiadiazolidine may has application(s) to

control tumorigenic responses, where arrest of cell cycle followed by cell death might have some potential therapeutic value to regress tumor. These possibilities require further investigation in detail. The multiple effects of P_3-25 on inhibition of NF-κB and in induction of cell death might be important for P_3-25's potential therapeutic value in NF-κB-driven inflammatory and tumorigenic responses.

Acknowledgements

This work was supported by the Department of Biotechnology (DBT), Govt. of India and core grant of Centre for DNA Fingerprinting and Diagnostics (CDFD). We duly acknowledge Prof. K.K. Narang, Banaras Hindu University, Varanasi, India for providing the thiadiazolidine derivatives.

References

[1] Abdel-Halin AM, Abdel-Aziz RM, El-Dein HS, El-Kafrawy AF. Synthesis and biological activity of some 2-[Pyrimidin-2-yl] imino-thiazoalidin-4-one. Ind J Heteroc Chem 1994;4:45–50.
[2] Geard C, Bernard B, Bertin E, Cristina De A, Simon LC, Dumas M, et al. Synthesis and biological activity of nitro heterocycles analogous to mega zol, a tryopanocidal lead. J Med Chem 2003;46:427–40.
[3] Choubey AK, Tripathi AK, Singh R. Synthesis, antifungal and antibacterial activities of some new 2-benzylideneamino-5-arylimino-3-oxo-1,2,4-thiadiazolidines. Ind J Chem 1998;37B:145–50.
[4] Manna P, Singh R, Narang KK, Manna SK. Synthesis, anti-fungal, antitumor and anti-inflammatory activities of some new 5-substitutedarylimino-2-N-p-tolylsulphonyl-3-oxo-1,2,4-thiadiazolidines. Ind J Heteroc Chem 2004;13:249–52.
[5] Manna P, Narang KK, Manna SK. 1,2,4-thiadiazolidine derivative inhibits Nuclear Transcription Factor-kappaB and its dependent genes activation but induces apoptosis. Int J Cancer 2005;113:549–60.
[6] Manna SK, Manna P, Sarkar A. Inhibition of RelA phosphorylation sensitizes chemotherapeutic agents-mediated apoptosis in constitutive NF-kappaB-expressing and chemoresistant cells. Cell Death Different 2007;4:158–70.
[7] Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 2005;5:749–59.
[8] Baeuerle PA, Baichwal VR. NF- κB as a frequent target for immuno-suppressive and anti-inflammatory molecules. Adv Immunol 1997;65:111–37.
[9] Mateyak MK, Obaya AJ, Sedivy JM. c-Myc regulates Cyclin D-Cdk4 and -Cdk6 activity but affects cell cycle progression at multiple independent points. Mol Cell Biol 1999;19:4672–83.
[10] Bouchard C, Thieke K, Maier A, Saffrich R, Hanley-Hyde J, Ansorge W, et al. Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. EMBO J 1999;18:5321–33.
[11] Galaktionov K, Chen X, Beach D. Cdc25 cell-cycle phosphatase as a target of c-myc. Nature 1996;382:511–7.
[12] Claassen GF, Hann SR. Myc-mediated transformation: the repression connection. Oncogene 1999;18:2925–33.
[13] Warner BJ, Blain SW, Seoane J, Massagué J. Myc downregulation by transforming growth factor β required for activation of the p15(Ink4b) G(1) arrest pathway. Mol Cell Biol 1999;19:5913–22.
[14] Bindels EMJ, Lallemand F, Balkenende A, Verwoerd D, Michalides R. Involvement of G1/S cyclins in estrogen-independent proliferation of estrogen receptor-positive breast cancer cells. Oncogene 2002;21:8158–65.
[15] Vanzulli S, Efeyan A, Benavides F, Helguero LA, Peters G, Shen J, et al. p21, p27 and p53 in estrogen and antiprogestin-induced tumor regression of experimental mouse mammary ductal carcinomas. Carcinogenesis 2002;23:749–58.
[16] Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 1999;96:857–68.
[17] Khwaja A. Apoptosis: Akt is more than just a Bad kinase. Nature 1999;401:33–4.
[18] Scheid MP, Woodgett JR. PKB/AKT: functional insights from genetic models. Nat Rev Mol Cell Biol 2001;2:760–8.
[19] Brognard J, Clark AS, Ni Y, Dennis PA. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 2001;61:3986–97.
[20] Zou X, Tsutsui T, Ray D, Blomquist JF, Ichijo H, Ucker DS, et al. The cell cycle-regulatory CDC25A phosphatase inhibits apoptosis signal-regulating kinase 1. Mol Cell Biol 2001;21:4818–28.
[21] Chen YR, Wang X, Templeton D, Davis RJ, Tan TH. The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. J Biol Chem 1996;271:31929–36.
[22] Raghavendra PB, Sreenivasan Y, Ramesh GT, Manna SK. Cardiac glycoside induces cell death via FasL by activating calcineurin and NF-AT, but apoptosis initially proceeds through activation of caspases. Apoptosis 2007;12:307–18.
[23] Shang Y, Baumrucker CR, Green MH. c-Myc is a major mediator of the synergistic growth inhibitory effects of retinoic acid and interferon in breast cancer cells. J Biol Chem 1998;273:30608–13.
[24] Raghavendra PB, Sreenivasan Y, Manna SK. Oleandrin induces apoptosis in human, but not in murine cells: dephosphorylation of Akt, expression of FasL, and alteration of membrane fluidity. Mol Immunol 2007;44:2292–302.
[25] Sarkar A, Sreenivasan Y, Ramesh GT, Manna SK. Beta-D-glucoside suppresses TNF-induced activation of nuclear transcription factor kappaB but potentiates apoptosis. J Biol Chem 2004;279:33768–81.
[26] Hernandez S, Hernandez L, Bea S, Cazorla M, Fernandez PL, Nadal A, et al. cdc25 cell cycle-activating phosphatases and c-myc expression in human non-Hodgkin's lymphomas. Cancer Res 1998;58:1762–7.
[27] Hughes PD, Belz GT, Fortner KA, Budd RC, Strasser A, Bouillet P. Apoptosis regulators Bim and Fas cooperate in shutdown of chronic immune responses and prevention of autoimmunity. Immunity 2008;28:197–205.
[28] Manna SK, Gangadharan C. Decrease in RelA phosphorylation by inhibiting protein kinase A induces cell death in NF-kappaB-expressing and drug-resistant tumor cells. Mol Immunol 2009;46:1340–50.