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LXR-α selectively reprogrammes cancer cells to enter into apoptosis

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Abstract There exists a general recognition of the fact that LXR-α, being a member of the nuclear receptor family, plays a crucial role in the biological process that connects inflammation, cholesterol homeostasis, and cellular decisions. In this context the present study was addressed to understand the role of LXR-α gene in the selective and specific reprogramming of cancer cells into a state of apoptosis leaving the normal cells unaffected. The results of this study revealed that LXR-α gene when activated in cancerous cells of diverse origin results in the regulation of genes coding for Bcl-2, AATF, and Par-4 in a fashion, forcing these cells to enter into the state of apoptosis leaving the normal cells unaffected. On the basis of this study we propose that in near future LXR-α agonist (Withaferin A) may definitely find its use in the therapeutic interventions directed towards the treatment of cancer.

Keywords Withaferin A · LXR-α · AATF · T cell leukemia · Apoptosis

Introduction

There has been forging evidence indicating a tantalizing link between cholesterol and cancer. More than a century ago, it was suggested that cancer was due to crystallization of cholesterol from living cells [1]. With due course of time, cholesterol was observed to accumulate in tumors [2], which suggested that cholesterol may in some way or the other, be associated with the regulation of proliferation. Similarly, there is growing evidence for a crucial connection between inflammation and carcinogenesis. There is an age old dictum by Rudolf Virchow, which suggests that the "lymphoreticular infiltrate" reflects the origin of cancer at the sites of chronic inflammation [3]. It can then be rightly said that if genetic damage is the "match that lights the fire" of cancer, some types of inflammation may provide the "fuel that feeds the flames". Nuclear factor kappa B (NF-kappa B) transcription factors are important in integrating multiple stress stimuli and regulate innate and adaptive immune responses seen at the states of inflammation [4]. With the recognition that inflammatory conditions are often associated with or precede cancer, it is natural to suspect a link between NF-kappa B and cancer [5, 6]. After integrating the two phenomenon common to a cancerous cell such as cholesterol homeostasis and inflammation, an important ligand activated nuclear receptor/transcription factor gains significance, called LXR-α which stands at the crossroads of both inflammation (by regulating NF-kappa B) [7] and cholesterol homeostasis (by regulating SREBP-1c) [8].

It is in this respect, it becomes pertinent enough to understand the molecular link which might exist linking LXR-α with inflammation and cholesterol homeostasis in respect to cancer in general and to derive a mechanistic procedure which a cancerous cell might adopt to reprogramme it into apoptotic state, by selectively activating LXR-α in the cancerous cells, using a specific ligand, i.e., Withaferin A [9]. Recent evidences have suggested that Withaferin A, a dietary steroidal lactone specifically induces apoptosis in cancer cells, leads to inhibition of

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NF-kappa B and also acts as a specific ligand for LXR-α [9–11]. So it might be that it is through LXR-α that its specific ligand can specifically target cancer cells. In order to get a holistic view of LXR-α regulating the apoptotic arm of cell cycle, it is imperative to understand how LXR-α nuclear receptor/transcription factor exert its control on the genes recognized to play crucial role in the control of cellular apoptosis, proliferation and quiescence processes in general.

Our recent investigations have clearly proposed pathways linking cell cycle with apoptosis based on the interaction between AATF, c-myc, Rb, and E2F [12] and the other linking cell cycle with quiescence based on the interaction between E2F-1 and Mxi1 which is known to maintain the cell in the quiescent state [13]. In accordance with the fact that Liver X Receptors (LXRs) are nuclear transcription factors which dimerize with the retinoid X Receptor (RXR) and, upon ligand binding, might regulate the expression of target genes such as the genes involved in cell cycle progression through NF-kappa B, our study was addressed to understand the following issues: (i) What is the effect of specific ligand activated LXR-α on the cellular state of various cancer specific cell lines? (ii) How ligand activated LXR-α affects the expression of genes coding for c-myc, par-4, AATF, Mxi1, Bcl-2, and E2F-1? (iii) Whether specific and selective downregulation of LXR-α gene within T cell leukemia cell line (Jurkat) can lead to opposite phenotypes to that observed with the specific activation of LXR-α in Jurkat cells by means of ligands used such as Withaferin A. Withaferin A is a bioactive steroidal lactone purified from the medicinal plant *Withania somnifera* (commonly known as ashwagandha or Indian winter cherry) [14] which has been recently recognized to be a ligand for LXR-α activation along with the known natural ligand such as 22(R) hydroxycholesterol [15].

Methods

Cell line and reagents

Human T cell leukemic cell line Jurkat, cervical cancer specific epithelial cell line Hela-229, human glioblastoma cells line U87, human B lymphoblastic cell line, human T cell leukemia cell line Molt-4, human monocytic cell line THP-1, and human neuroblastoma cell line IMR-32 (obtained from National centre for cell science, Pune) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), cells were counted in hemocytometer chamber and viability was determined by Trypan Blue dye exclusion. For morphological assessment of cells, the cytospin slides were subjected to Leishman stain and differential counts were performed under light microscopy on a minimum of hundred cells for each experiment. Withaferin A was obtained from Merck, Germany and Revert Aid^{TM} first strand cDNA synthesis kit was obtained from Fermentas, Maryland. siRNA specific for LXR-α was obtained from Santa Cruz, California and lipofectamine 2000 Transfection reagent was obtained from Invitrogen, California.

Normal peripheral blood mononuclear cell isolation

Human normal peripheral blood mononuclear cells (PBMCs) were obtained from normal healthy volunteers, who were fasting for 12 h or were abstained from any medication for 2 weeks before blood donation. Blood was drawn through venipuncture into heparinized tubes and PBMCs were isolated using density gradient centrifugation method. Briefly, 5 ml of heparinized blood was gently layered onto 4 ml of Histopaque (Sigma-Aldrich, Missouri; solution containing polysucrose and sodium diatrizoate, adjusted to a density of 1.077 ± 0.001 g/ml) and centrifuged at 400×*g* in swinging bucket rotor, for 30 min at room temperature. After centrifugation, the layer of mononuclear cells formed as an opaque ring at the plasma-Histopaque interface was carefully picked up with a Pasteur pipette. These PBMCs were washed twice with phosphate buffered saline at 250×*g* for 10 min and then subjected to purification and enrichment of T lymphocytes.

T cell purification

T cells from PBMCs were purified using Pan T cell isolation kit by depletion of non-T cells (negative selection). Non-T cells, i.e., B cells, NK cells, dendritic cells, monocytes, granulocytes, and erythroid cells were indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies against CD14, CD16, CD19, CD36, CD56, CD123 and glycophorin A and Anti-Biotin Microbeads. Isolation of highly pure T cells was achieved by depletion of magnetic labeled cells.

Cell culture and transfection experiments

In the first set of experiment, Jurkat CD4+ T cells and human peripheral blood mononuclear cells separated by histopaque obtained from sigma, were seeded in 24-well culture plates at an initial density of about 0.5×10^5 cells per well in RPMI-1640 medium containing 10% FCS at 37°C in 5% CO_2 atmosphere. After 24 h synchronization these cells were treated with the addition of Withaferin A (10 μM) obtained from Merck and 22(R) hydroxycholesterol (10 μM) obtained from Sigma. These treated cells were incubated at 37°C for 24 h in 5% CO_2 atmosphere.

At the end of this incubation period the cells from each well were harvested and processed further for apoptosis assay. Each experiment was done in triplicate to assess the reproducibility of results.

In second set of experiment, Jurkat CD4+ T cells and normal T cells separated as explained earlier along with Jurkat, Hela-229, MOLT-4, EB-3, and human glioblastoma U87 cell lines were seeded in 24-well culture plates as described earlier. After 24 h synchronization these cells were harvested and processed further for the isolation of total RNA to check the transcriptional expression of LXR-α gene in all the cell lines studied.

In the third set of experiment, cancerous cells from all the cell lines mentioned were seeded in 24-well culture plates as described earlier. After 24 h synchronization these cells were treated with the addition of Withaferin A (10 μM) obtained from Merck, Germany. These treated cells were incubated at 37°C for 24 h in 5% CO_2 atmosphere. At the end of this incubation period the cells from each well were harvested and processed further for apoptosis assay.

In fourth set of experiment, Jurkat CD4+ T cells were seeded in 24-well culture plates as described earlier. After 24 h synchronization these cells were treated with the addition of Withaferin A (0–12 μM) obtained from Merck, Germany. At the end of 24 h incubation period the cells from each well were harvested and processed further for apoptosis assay.

In fifth set of experiment, Jurkat CD4+ T cells were seeded in 24-well culture plates as described earlier. After 24 h synchronization these cells were treated with the addition of Withaferin A (10 μM) obtained from Merck, Germany. At the end of this incubation period the cells from each well were harvested and processed further for the apoptosis assay.

In sixth set of experiment, Jurkat CD4+ T cells were seeded in 24-well culture plates as described earlier. After 24 h synchronization these cells were treated with the addition of Withaferin A (10 μM) obtained from Merck, Germany. At the end of 24 h incubation period the cells from each well were harvested and processed further for the isolation of total RNA and protein.

In the seventh set of experiment, Jurkat CD4+ T cells were seeded in 24-well culture plates as described earlier. After 24 h synchronization these cells were transfected with the siRNA specific for LXR-α. At the end of 36 h incubation period the cells from each well were harvested and processed further for the isolation of total RNA and protein as well as cell cycle analysis. Also in order to perform the growth curve analysis the LXR-α siRNA transfected cells were harvested at different time intervals (0–36 h) and processed for FACS analysis by staining the cells with propidium iodide (1 mg/ml).

Each of these experiments were done in triplicate to assess the reproducibility of results.

Plasmid transfection experiment

Bioinformatics analysis of Mxi1 gene promoter (using JASPAR and TF search software) revealed the existence of SREBP-1c response elements. In order to ascertain whether or not Withaferin A, which is a ligand for LXR-α finally leading to the activation of SREBP-1c [8] could affect Mxi-1 gene expression, the reporter cassette comprising of SREBP-1c response element (present on Mxi1 promoter) with the following sequence 5′ GTGGGGGGAT 3′ was designed and incorporated in β-gal reporter plasmid with the help of TOPO-cloning reaction as per manufacturer's instructions. TOPO vector containing scrambled sequence was used as control. Jurkat CD4+ T cells were seeded in 24-well culture plates at an initial density of about 0.5×10^5 cells per well in RPMI-1640 medium containing 10% FCS at 37°C in 5% CO_2 atmosphere. After 24 h synchronization these cells were transfected with the reporter plasmid using Lipofectamine 2000 transfection reagent from Invitrogen, California. These transfected cells were exposed to medium containing either no stimulus or Withaferin A (10 μM) up to 24 h at 37°C in 5% CO_2 atmosphere. At the end of incubation period, the cells were lysed and supernatants were assayed for β-gal reporter activity as per the procedure reported earlier [16]. To further provide an evidence that Withaferin A effect on Jurkat cells apoptosis is through LXR-α another reporter assay was performed using TOPO® Reporter Kit (Invitrogen). This time, LXR-α response element was amplified by PCR and was incorporated into the pBlue TOPO® TA cloning vector by the manufacturer protocol. This vector containing the LXR-α response element was transfected to the cultured Jurkat cells as described earlier. After 6 h of transfection, cells were exposed to Withaferin A (10 μM) for 24 h. β-galactosidase activity was measure by β-Gal Assay Kit (Invitrogen). The experiments were repeated thrice and results reported as relative β-gal activity.

In another set of experiment, Jurkat CD4+ T cells were seeded in 24-well culture plates at an initial density of about 0.5×10^5 cells per well in RPMI-1640 medium containing 10% FCS at 37°C in 5% CO_2 atmosphere. After 24 h synchronization these cells were transfected cells with the reporter plasmid using Lipofectamine 2000 transfection reagent (Invitrogen). In order to validate the finding that E2F-1 gene has a direct role in regulating Mxi1 expression [13] the plasmid transfected Jurkat cells were co-transfected with E2F-1 specific siRNA from Santa Cruz also by again using Lipofectamine 2000 (Invitrogen). After this the cells were incubated for 24 h at 37°C in 5% CO_2 atmosphere. At the end of incubation period, the cells were lysed and

supernatants were assayed for β-gal reporter activity as per the procedure reported earlier [16]. The experiments were repeated thrice and results reported as relative β-gal activity.

Gene expression at transcriptional level

Total RNA was extracted by using standard method [17]. The integrity of RNA was verified by electrophoretic size separation in 1% ethidium bromide stained agarose gels. cDNA for total RNA was synthesized using Revert Aid Tm cDNA synthesis kit (Fermentas, Maryland). Genes coding for AATF, Par-4, c-myc, LXR-α, and β-actin were amplified using oligonucleotide primers:

GACACGGACAAAAGGTATTGCG and AGACCCA GTCCCTCTGAATCT for AATF gene, CTCTGGTTTCA AGTAGCACACTG and TGACCCACAACTTTCAAAA GAGT for Par-4 gene, CCAGCAGCGACTCTGAGG and CCAAGACGTTGTGTGTTC for cmyc gene, CAGATTG CCCTGCTGAAGAC and GAACTCGAAGATGGGGT TGA for LXR-α, and CATGTACGTTGCATCCAGGC and CTCCTTAATGTCACGCACGAT for β-actin gene with Taq PCR core kit (Qiagen) along with standardized log phase amplification programmes. β-Actin gene expression was used as a control for RNA loading and RT efficiency. Reaction products were resolved on 2% ethidium bromide stained agarose gels followed by densitometric scanning of each band on the gels. Intensity ratio of target mRNA to β-actin mRNA for each gene was expressed as percentage of that in untreated Jurkat cells. All the oligonucleotide primers were designed using Primer 3 software.

Gene expression at translational level

In order to assess the translational profile of the cells, the control as well as Withaferin A treated Jurkat cells were harvested in Laemmli sample buffer (10% 2-mercaptoethanol, 6% SDS, 20% glycerol, 0.2 mg/ml bromophenol blue). After lysis the proteins from the above cellular extracts were separated by SDS-PAGE on 12.5% polyacrylamide gels. Proteins were then transferred electrophoretically onto nitrocellulose membranes (0.45 μM). Non-specific sites were blocked by incubating membranes (1 h, room temperature) in 5% (w/v) non-fat milk powder in phosphate-buffered saline (PBS) containing 0.05% Tween 20. Membranes were probed with primary antibodies against α-actin, c-Myc, Mxi1, Bcl-2, LXR-α, and E2F-1 using standardized dilutions for 2 h at 37°C. All the antibodies were obtained from Santa Cruz Biotechnology, California. Membranes were then washed with PBS containing 0.05% Tween 20 and incubated with alkaline phosphatase conjugated secondary IgG antibody (1:2500) for 1 h at 37°C. After this membranes were washed with PBS containing 0.05% Tween 20 and followed with the addition of BCIP/NBT substrate for alkaline phosphatase activity. Bands visualized on addition of substrate were analyzed semi-quantitatively using SCION IMAGE analysis software involving densitometric variations.

Cell cycle and apoptosis assay

The LXR-α knockdown as well as control Jurkat cells were subjected to cell cycle analysis for which the cells were harvested and washed with PBS and kept in 70% ethanol overnight at −20°C. After this the cells were centrifuged at 1000 rpm for 5 min, suspended in PBS and treated with propidium iodide (1 mg/ml, Sigma-Aldrich, Missouri). After an incubation period of 30 min in dark, the cells were proceeded for analysis using Becton–Dickinson FACS caliber using Cell quest software upon the acquisition of 10,000 cells for the final analysis. In order to study the growth curve of Jurkat cells after LXR-α siRNA transfection, the same protocol for cell cycle analysis was followed as mentioned above at different time intervals (0–36 h).

For studying the survival and apoptosis of the control (1% DMSO treated), Withaferin A treated cells and 22(R) hydroxycholesterol treated cells, the cells were harvested and washed with PBS followed by suspension of cells in 1× binding buffer containing Annexin-V FITC conjugated and propidium iodide for 20 min. After the incubation period the cells were proceeded for analysis on the FACS caliber with an acquisition of 10,000 cells.

Cellular ultrastructural study

In order to explore the ultrastructural features within the Jurkat cells derived from the control and Withaferin A treated culture wells, the cells were processed for transmission electron microscopic examination using the following method:

The cells were fixed in 3% buffered glutaraldehyde, and put in Sorensen's buffer with sucrose for 15 min. Later the sections were post-fixed in 1% osmium tetroxide for 1 h and dehydrated in 50, 70, 90, and 100% alcohol for 30 min each. After this the cells were given two changes of propylene oxide for 15 min each after which the cells were embedded in Epon resin. Ultrathin sections were cut from the resin molds and the best representative sections were mounted on copper grids, stained with uranyl acetate and lead citrate and examined with Zeiss 906 electron microscope. Ultrathin sections were made using a Reichert ultramicrotome. For the analysis of these ultrathin sections, 12 respective cells from different representative fields were chosen to access the apoptotic features of chromatin fragmentation along with nuclear and plasma membrane disintegration processes characteristic of an apoptotic cell in both the control and Withaferin A (10 μM) treated Jurkat cells.

Statistical analysis

Data were expressed as mean ± S.D. of each experiment done in triplicate. The P value <0.05 was considered statistically significant.

Results

Ligand activated LXR-α and its effect on the cellular survival/apoptosis

The first set of experiments were designed to assess whether or not Withaferin A, a newly recognized ligand for LXR-α has similar effects on Jurkat cells as the known ligand 22(R) hydroxycholesterol has. For this Jurkat cells were individually treated with Withaferin A and 22(R) hydroxycholesterol and the results revealed that the amount of apoptosis analyzed in both the cases was nearly the same which amounted to approximately 50% in case of Withaferin A treated cells and to about 43% in case of 22(R) hydroxycholesterol treated Jurkat cells (Fig. 1). Further treatment of normal peripheral blood mononuclear cells with the same concentrations of 10 μM each of Withaferin A and 22(R) hydroxycholesterol revealed that both the ligands for LXR-α activation specifically and selectively affected only the cancerous cells but not the normal cells, i.e., the PBMCs containing both the lymphocytic and monocytic populations, respectively (Fig. 1), where there was no appreciable change in the apoptotic index of control

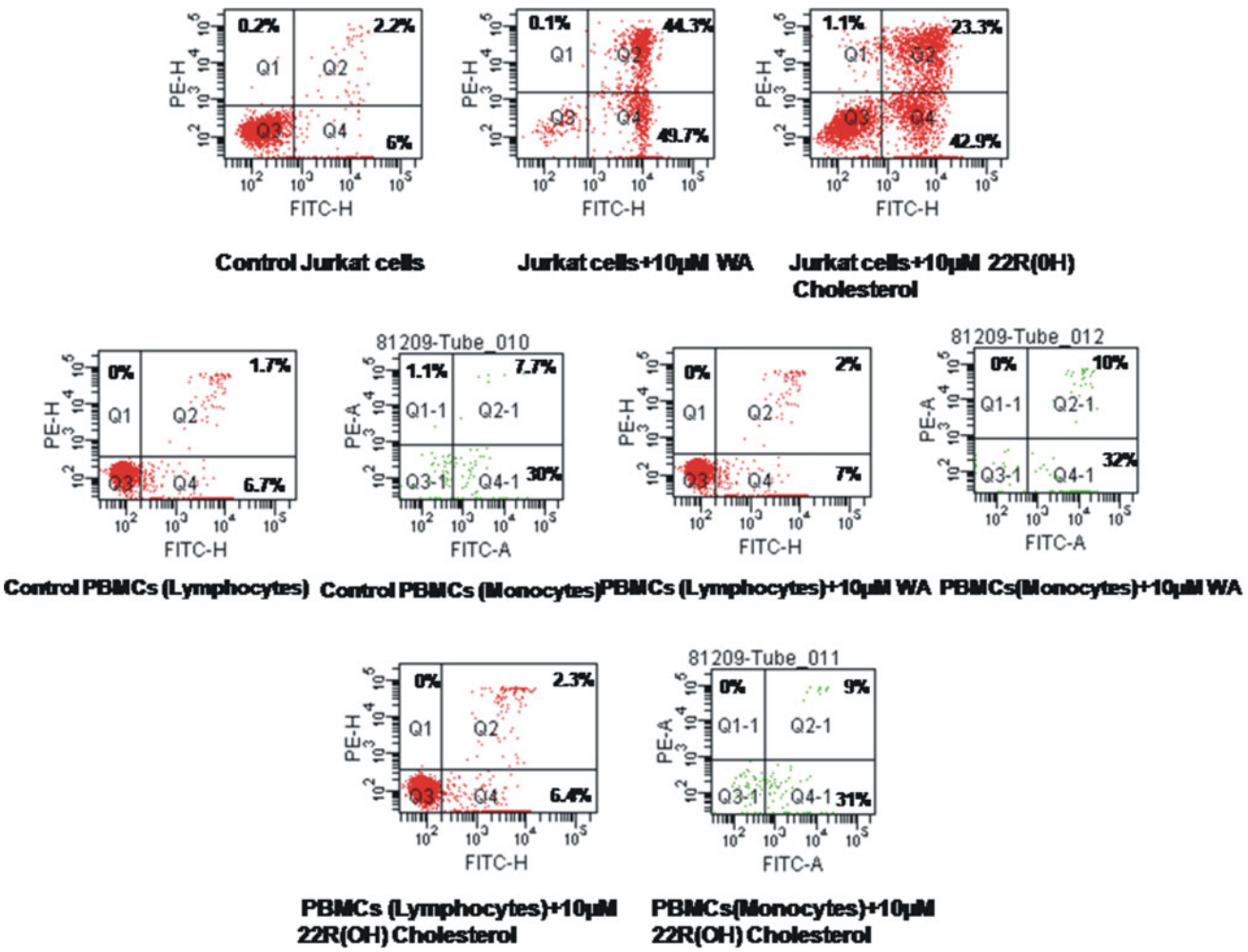

Fig. 1 Representative FACS analysis of cellular survival coupled with apoptosis in Withaferin A and 22R(OH) cholesterol treated Jurkat cells as compared to that observed in the control Jurkat cells as well as normal monocytes and lymphocytes. Each experiment was done in triplicate for reproducibility. The *percentage values* indicated in the figure represent the percentage of cells present in the quadrants representative of the apoptotic stage of a cell. The *upper right quadrant* represents the percentage of cells in late apoptotic phase; the *lower right quadrant* represents the percentage of cells in the early apoptotic phase; the *upper left* indicates the percentage of cells which are only propidium iodide stained, i.e., necrotic or dead cells

as well as treated cells. Also LXR-α expression when compared with normal T cells and T cell Leukemia Jurkat cells revealed no marked difference in the expression level in both the cells studied (Fig. 2). Also in order to compare LXR-α protein expression in normal T cells and Jurkat cells after treatment with Withaferin A, Fig. 3 revealed that after treatment there was a relative increase in LXR-α at protein level in Jurkat cells, when compared with T cells (Fig. 3). In order to access the relative mRNA expression level of LXR-α in the various cell lines studied irrespective of the type of cancer, Fig. 4 demonstrated that, however, the expression of LXR-α in glioblastoma U87 cells is much higher than that found in other cell lines employed in the study.

In the second set of experiments cells from various unrelated cancer cell lines such as Hela-229, U87 glioblastoma cells, EB-3, Molt-4, THP-1, IMR-32, and Jurkat cells were individually treated with Withaferin A at 10 μM concentration and analyzed for apoptotic index of these cells. The results of this particular experiment revealed that irrespective of the cancerous cell line being employed in the present study the effect of Withaferin A was almost similar in all the cancerous cells in question. There was a marked increase in the apoptotic percentage of cells in the various cell lines treated with Withaferin A as compared to control cells in each case (Fig. 5).

In another set of experiments, a dose-dependent (Fig. 6) and time-dependent (Fig. 7) study was conducted with respect to Withaferin A taking Jurkat cells as archetype cellular model systems. The results of such an experiment unambiguously revealed that increasing doses (0–12 μM) of Withaferin A will lead to increasing apoptotic percentage of cells with maximum apoptosis occurring with 10 μM concentration (Fig. 6). Also a time-dependent analysis revealed that with increasing time intervals (6–48 h) of cells treated with 10 μM concentration of Withaferin A there was found to be a large percentage of cells (approximately 75% at 48 h) in apoptotic phase of cell cycle (Fig. 7).

Since, the above-mentioned findings indicated a potential role of ligand (Withaferin A) activated LXR-α gene in cellular apoptotic process an attempt was made to perform a cytometric analysis of the control as well as Withaferin A treated Jurkat cells at ultrastructural level. The electron microscopic analysis of these cells revealed that almost every cell in the Withaferin A treated Jurkat cells showed typical features associated with apoptotic process but in contrast the control cells showed normal

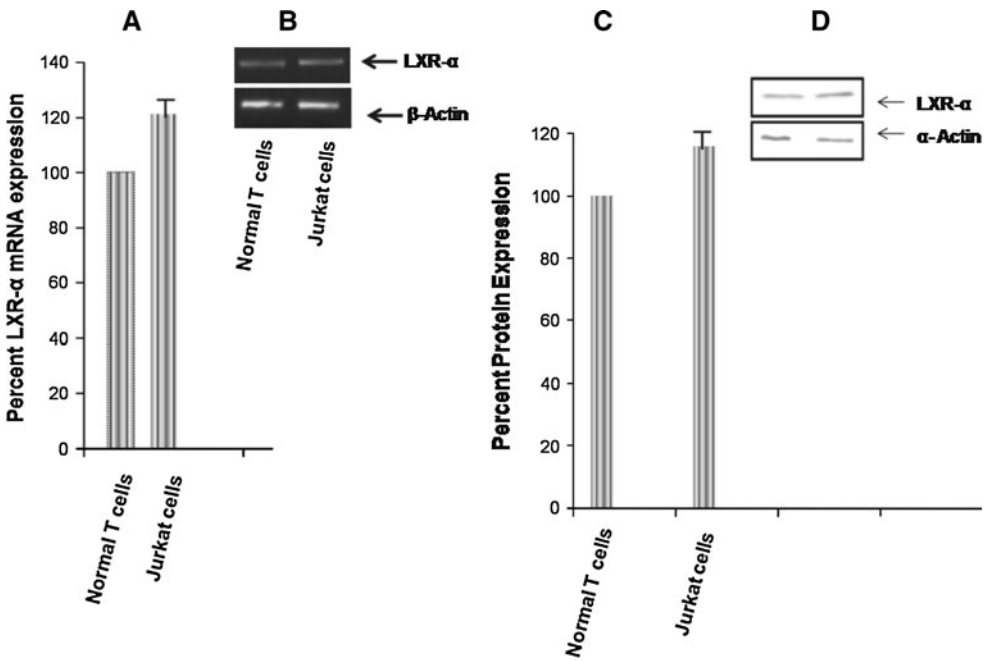

Fig. 2 **a** Transcriptional expression of LXR-α gene in normal T cells and Jurkat cells. mRNA expression of the gene was normalized by *β*-actin mRNA expression. Each *bar* represents mean ± S.D. of the experiment done in triplicate. **b** Representative agarose gel photographs showing ethidium bromide stained RT-PCR products in normal T cells and Jurkat cells. **c** Translational expression of gene coding for LXR-α in normal T cells and Jurkat cells. Protein expression of every gene was normalized by the expression of α-actin protein expression via western blotting. Each *bar* represents mean ± S.D. of the experiment done in triplicate. **d** Representative western blot showing translational expression of gene coding for LXR-α in normal T cells and Jurkat cells

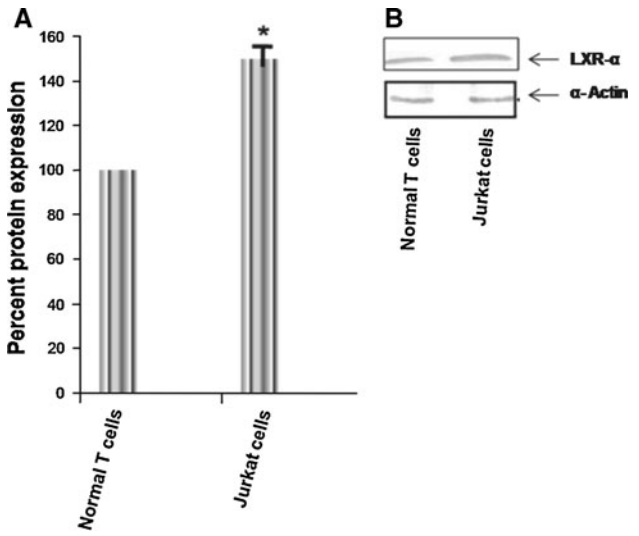

Fig. 3 **a** Translational expression of gene coding for LXR-α in Withaferin A treated (10 μM) normal T cells and Jurkat cells. Protein expression of every gene was normalized by the expression of α-actin protein expression via western blotting. Each *bar* represents mean ± S.D. of the experiment done in triplicate; *statistical significance at $P < 0.05$. **b** Representative western blot showing translational expression of gene coding for LXR-α in Withaferin A treated (10 μM) normal T cells and Jurkat cells

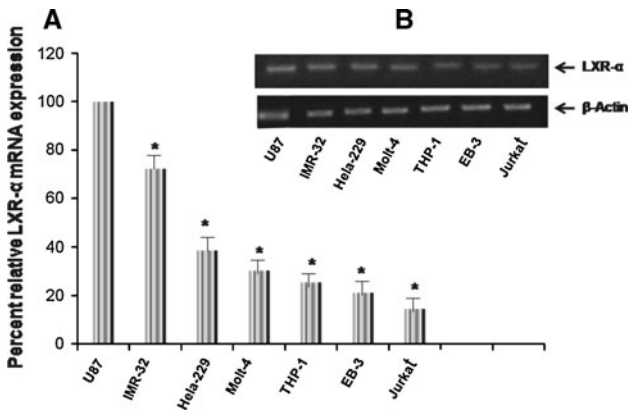

Fig. 4 **a** Relative mRNA expression of LXR-α gene in Jurkat, MOLT-4, EB-3, U87, Hela-229, IMR-32, and THP-1 cells with respect to U87 glioblastoma cells. mRNA expression of all the genes was normalized by β-actin mRNA expression. Each *bar* represents mean ± S.D. of the experiment done in triplicate; *statistical significance at $P < 0.05$. **b** Representative agarose gel photographs showing ethidium bromide stained RT-PCR products in all the cell lines employed

phenotypes based upon the pictures captured under the same magnification from different fields of each ultrathin section (Fig. 8a, b).

Bioinformatic analysis of the promoter region of Mxi1 gene (using JASPAR and TF search software) revealed the presence of SREBP-1c response element binding site on it. In order to analyze the functionality of SREBP-1c response element which is directly regulated by LXR-α gene [8], on Mxi1 promoter, a reporter plasmid assay was performed which revealed that SREBP-1c response element positively regulates Mxi1 gene expression as on Withaferin A treatment there was found to be an increase in the Mxi1 reporter activity (Fig. 9). Similar was the case observed on co-transfection of the Jurkat cells with E2F-1 siRNA and the plasmid containing SREBP-1c sequence conjugated to a reporter gene (Fig. 9) which clearly reveals that E2F-1 gene has an intrinsic down-regulatory effect towards the SREBP-1c response element present on Mxi1 promoter sequence. Also in order to provide a direct evidence of the apoptotic effect of Withaferin A on Jurkat cells through LXR-α another reporter assay was performed. In this we found a significant increase in the LXR-α response element dependent β-galactosidase activity in response to Withaferin A (Fig. 10).

In another set of experiments, when siRNA specific for LXR-α was transfected into Jurkat cells, the LXR-α knockdown cells exhibited increased proliferative potential as indicated by the cell cycle analysis of the LXR-α knockdown as well as control cells. It was indicated that when control Jurkat cells exhibited about 30% of the total cells in S phase of the cell cycle, the LXR-α knockdown Jurkat cells were about 62% in the S phase of cell cycle (Fig. 11). Also in order to support the fact that knocking down LXR-α increases cells proliferation we performed a time-dependent assay of LXR-α siRNA transfection (0–36 h) and then harvested the cells after every interval for cell cycle analysis. Figure 12 clearly reveals the percentage of cells in S phase of cell cycle with respect to each time interval showing maximum proliferation at 36 h of transfection of siRNA.

Cellular reprogramming of the genes involved in quiescence, apoptosis, or proliferation by ligand activated LXR-α

As compared to control cells, the LXR-α activated Jurkat cells exhibited significant decrease in the translational profile of genes coding for c-myc, E2F-1, and Bcl-2 which was accompanied by a marked increase in Mxi1 expression (Fig. 13). Furthermore, transcriptional profile of genes coding for AATF and c-myc showed a significant decrease in ligand activated LXR-α Jurkat cells as compared to control cells (Fig. 14) which was accompanied by a marked increase in transcriptional profile of genes coding for LXR-α and Par-4 gene (Fig. 14). The relative protein levels of Bcl-2 and c-myc after treatment with Withaferin A in normal PBMCs indicated that after LXR-α activation

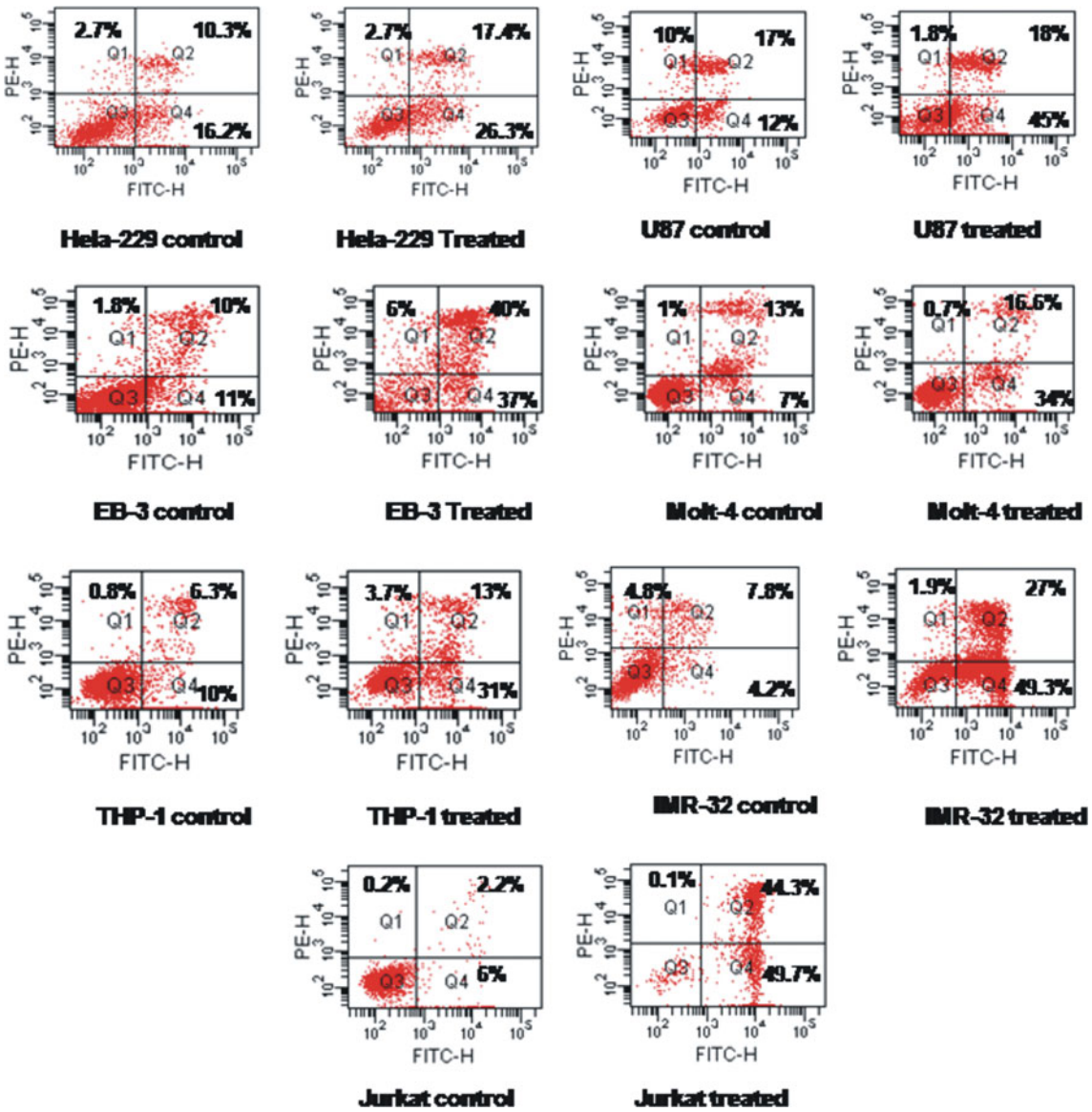

Fig. 5 Representative FACS analysis of cellular survival coupled with apoptosis in Withaferin A treated cell lines (Hela-229, U87 glioblastoma, EB-3, Molt-4, THP-1, IMR-32, and Jurkat) as compared to that observed in their respective control cells. Each experiment was done in triplicate for reproducibility. The *percentage values* indicated in the figure represent the percentage of cells present in the quadrants representative of the apoptotic stage of a cell. The *upper right quadrant* represents the percentage of cells in late apoptotic phase; the *lower right quadrant* represents the percentage of cells in the early apoptotic phase; the *upper left* indicates the percentage of cells which are only propidium iodide stained, i.e., necrotic or dead cells

via Withaferin A treatment there was no effect on c-myc expression but Bcl-2 protein level increased significantly (Fig. 15).

In another set of experiments, LXR-α expression was found to be significantly downregulated at translational level in LXR-α siRNA transfected Jurkat cells as compared to control cells (Fig. 16a). Similarly, the transcriptional profile of genes coding for AATF and c-myc also showed a marked increase in the LXR-α knockdown cells as compared to control cells (Fig. 16b).

Discussion

With due pertinence to the fact that in spite of all the treatment regimens available with medical sciences, still various developmental diseases such as cancer have not found a sure cure and the number of people getting afflicted by this particular disease are increasing day by day. A key treatment regimen to circumvent all the problems faced with the current protocols is to deal with the disease right at the basic cellular level. There have

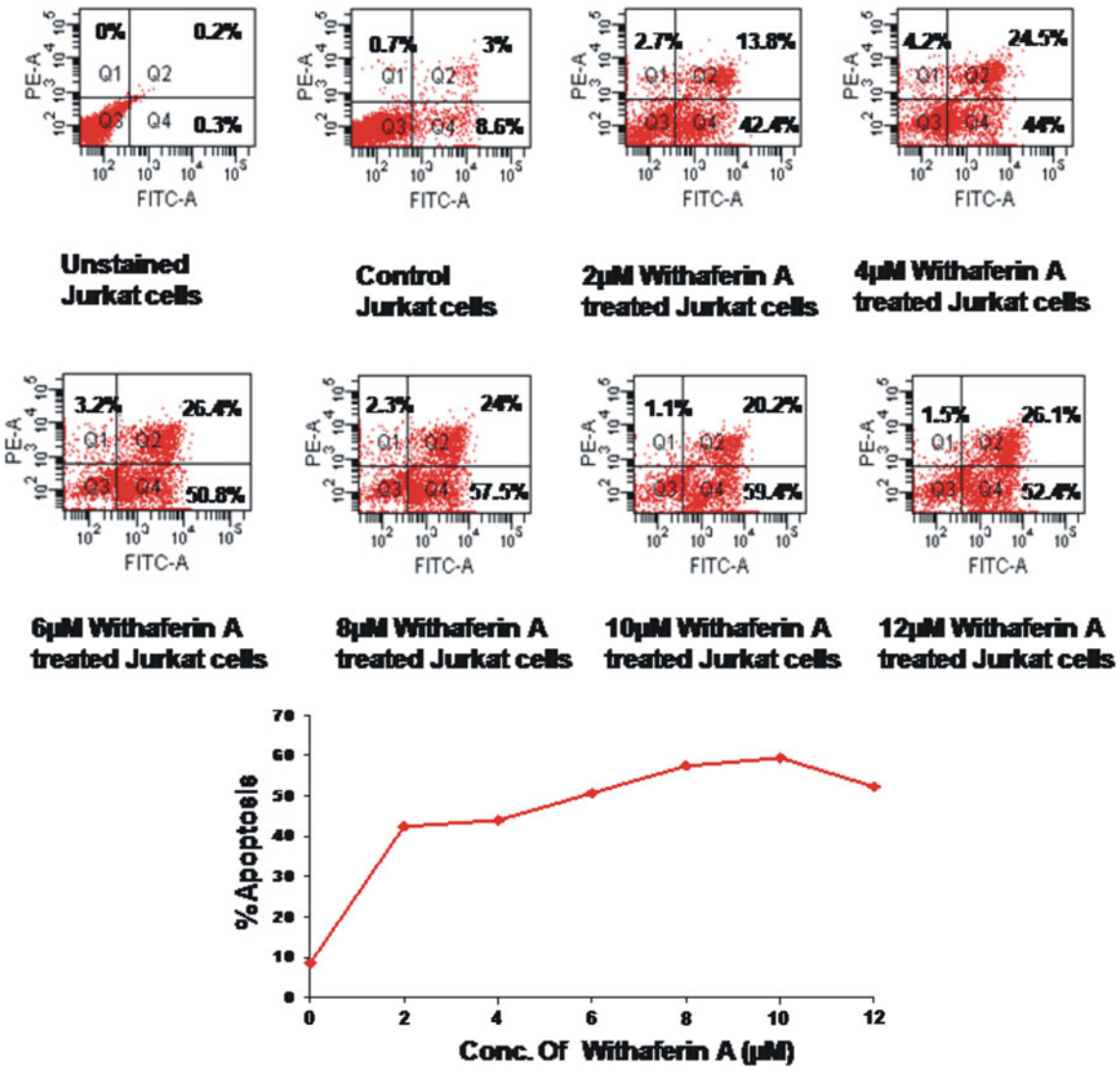

Fig. 6 Representative FACS analysis of cellular survival coupled with apoptosis in Withaferin A treated (dose-dependent manner: 2, 4, 6, 8, 10, and 12 μM) Jurkat cells as compared to that observed in their respective control cells. Each experiment was done in triplicate for reproducibility. The *percentage values* indicated in the figure represent the percentage of cells present in the quadrants representative of the apoptotic stage of a cell. The *upper right quadrant* represents the percentage of cells in late apoptotic phase; the *lower right quadrant* represents the percentage of cells in the early apoptotic phase; the *upper left* indicates the percentage of cells which are only propidium iodide stained, i.e., necrotic or dead cells

been reported evidences of the fact that a nuclear receptor/transcription factor, LXR-α on its activation reduced the proliferation and caused growth arrest in vascular smooth muscle cells [18]. Also there have been evidences suggesting the antiproliferative role of LXR-α in breast cancer cell lines [19]. In this study, we provide an evidence for a strong antiproliferative and apoptotic effect of LXR-α on specifically cancer cells irrespective of the type of cancer leaving the normal cells unaffected. This study with further experimental evidences can in near future provide the basis for the new treatment regimen which would be specifically against the cancerous cells without harming the normal cells in question.

As outlined in the "Introduction" section, cancer cells have been marked with increased cholesterol uptake required for the membrane biogenesis and LXR-α is an established sensor of intracellular cholesterol and lipid metabolism (important attributes of a cancerous cell), which become activated transcriptionally upon binding of certain oxysterols such as 22R(OH) cholesterol and Withaferin A, a steroidal lactone, the results reported here assume importance in relating LXR-α specifically with cancer cells and not affecting normal cells. It is through

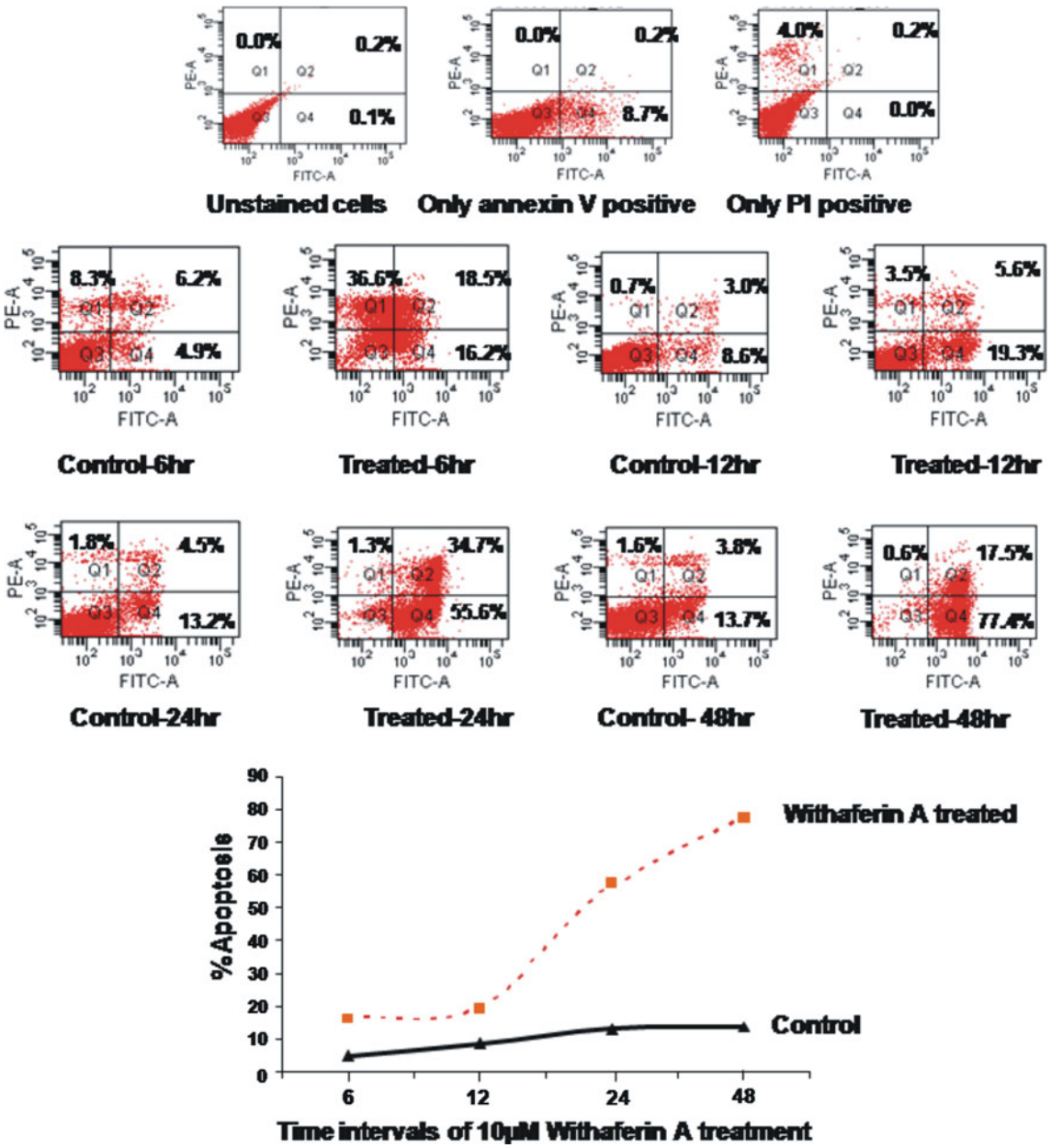

Fig. 7 Representative FACS analysis of cellular survival coupled with apoptosis in Withaferin A treated (time-dependent manner: 6, 12, 24, 48 h) Jurkat cells as compared to that observed in their respective control cells. Each experiment was done in triplicate for reproducibility. The *percentage values* indicated in the figure represent the percentage of cells present in the quadrants representative of the apoptotic stage of a cell. The *upper right quadrant* represents the percentage of cells in late apoptotic phase; the *lower right quadrant* represents the percentage of cells in the early apoptotic phase; the *upper left* indicates the percentage of cells which are only propidium iodide stained, i.e., necrotic or dead cells

this study only that the various decisions pertinent to a cell such as proliferation, apoptosis, and quiescence become evident through the ligand activated LXR-α targeted genes.

In accordance with the fact that 22(R) hydroxycholesterol is a well known natural ligand of LXR-α and Withaferin A is also a ligand for LXR-α [9, 20], we for the first time demonstrated that 22(R) hydroxycholesterol and Withaferin A both have similar activities in cancerous cells driving them towards the apoptotic state through LXR-α without having any appreciable effect on the normal monocytes and lymphocytes gated from the normal human peripheral blood mononuclear cells (Fig. 1). Thus, it emerged as an important observation in the

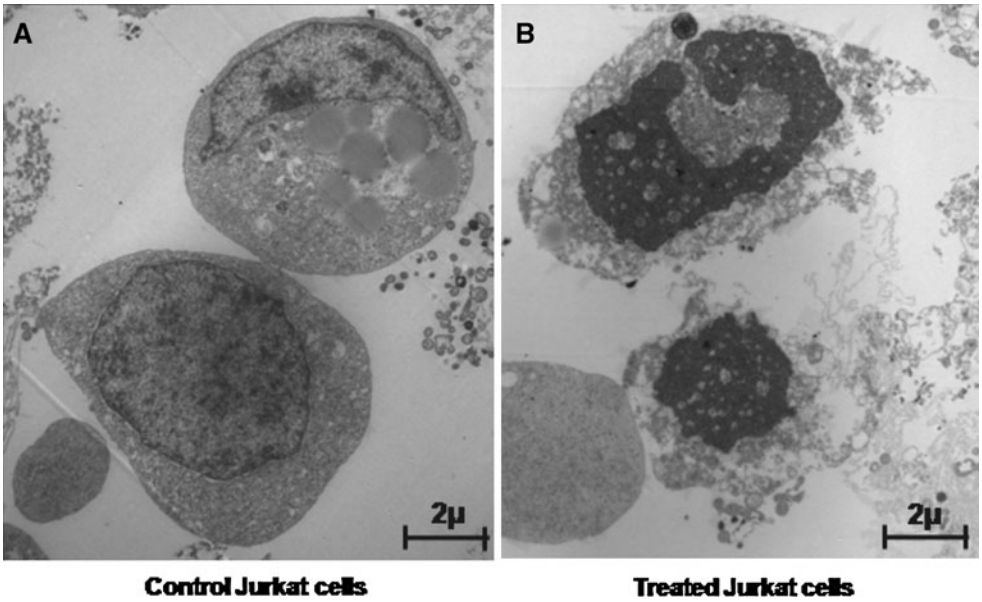

Fig. 8 Representative ultrastructural analysis of control and Withaferin A treated Jurkat cells, respectively, by Transmission Electron Microscopy (TEM)

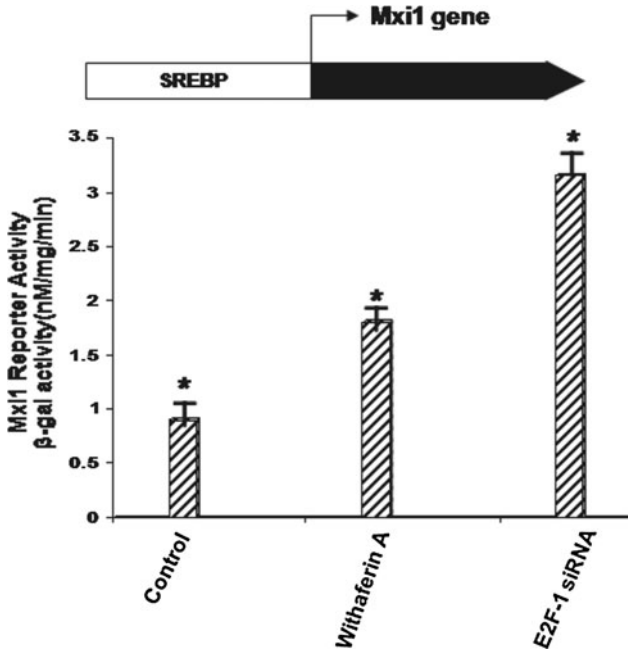

Fig. 9 Identification of SREBP-1c response element in the promoter region of Mxi1 gene using Bioinformatics tools and exploitation of these elements in understanding the regulation of Mxi1 gene using reporter plasmid and Jurkat T cell leukemia cell line as a cellular model. The representative figure shows the Mxi1 reporter activity within Jurkat cells exposed to either no stimulus or Withaferin A (10 μM) or E2F-1 siRNA. Each experiment was performed in triplicate for assessing the reproducibility

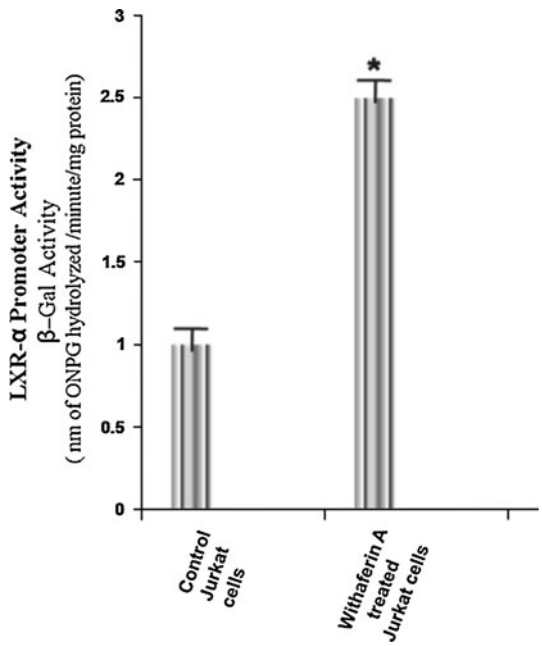

Fig. 10 LXR-α promoter dependent β-galactosidase reporter activity in Jurkat cells exposed to Withaferin A (10 μM). Each *bar* represents mean ± S.D. of the experiments done in triplicate (statistical significance is shown by $*P < 0.05$)

context of this particular study that these ligands for LXR-α show the specific apoptotic activity towards only the cancer cells without affecting the normal cells in question. In fact there was no marked difference in the relative expression level of LXR-α in normal T cells when compared with T cell leukemia cell line, Jurkat (Fig. 2), also LXR-α expression was evident in all the cancer cell lines studied with U87, Glioblastoma cells showing maximum LXR-α expression when compared

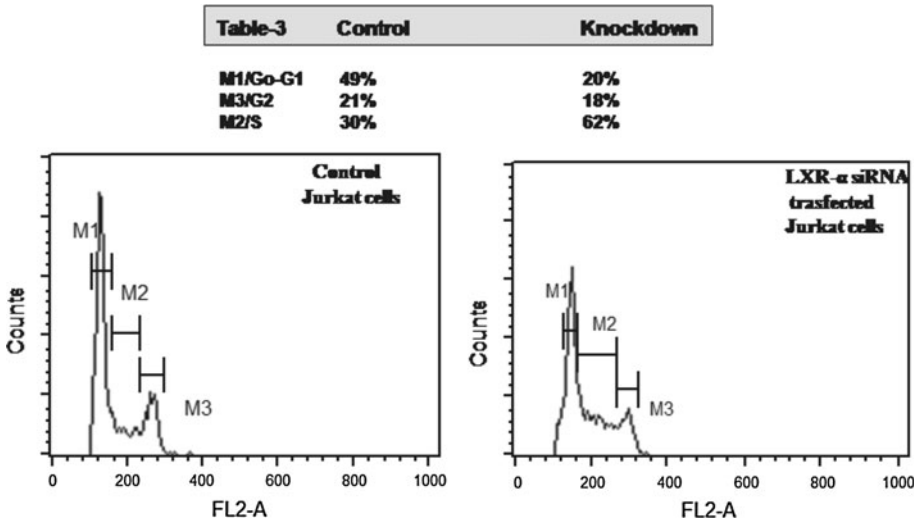

Fig. 11 Representative FACS cell cycle analysis of control and LXR-α siRNA transfected Jurkat cells, respectively. The *percentage values* indicated in the figure represent the percentage of cells in various stages of cell cycle

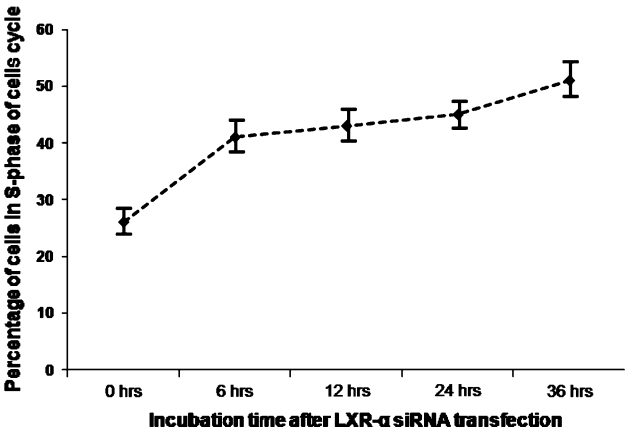

Fig. 12 Growth curve representing the percentage of cells in S phase of cell cycle after LXR-α siRNA transfection at different time intervals. Each *marked line* represents mean ± S.D. of the experiments done in triplicate

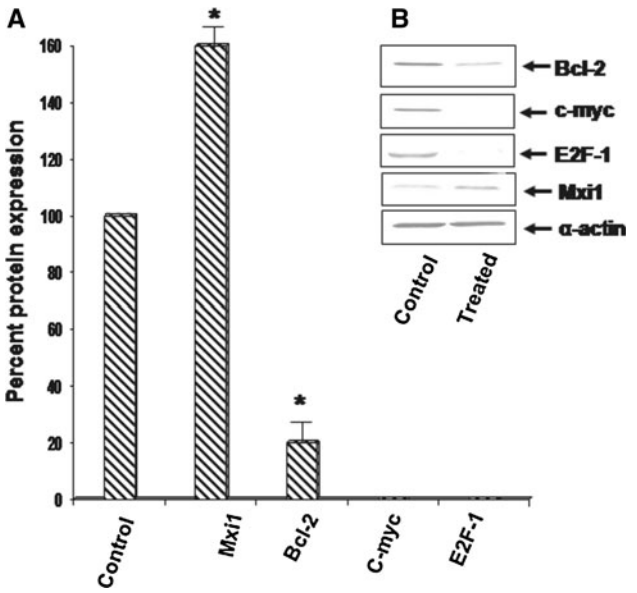

Fig. 13 **a** Translational expression of genes coding for E2F-1, c-Myc, Mxi1, and Bcl-2 in control and Withaferin A treated Jurkat cells. Protein expression of every gene was normalized by the expression of α-actin protein expression via western blotting. Each *bar* represents mean ± S.D. of the experiment done in triplicate; *statistical significance at $P < 0.05$. **b** Representative western blot showing translational expression of genes coding for E2F-1, c-Myc, Mxi, and Bcl-2 in control and Withaferin A treated Jurkat cells

with all the other cells lines employed (Fig. 4). This might be an indication to the fact that though LXR-α was expressed in all the cancer specific cell lines but it was not activated intrinsically due to the absence of specific ligands such as oxysterols in case of cancer cells pertaining to hypoxic conditions and absence of cholesterol oxidation. It was also evident from the results that irrespective of the various unrelated cancer cell lines used, Withaferin A had a devastating apoptotic effect on all the cancer cell lines studied especially in T cell leukemic Jurkat cells which were taken as archetype cellular models both in a dose- and time-dependent fashion, respectively (Figs. 5, 6, 7). We have recently demonstrated the RNomic control exerted by E2F-1 targeted genes on cellular quiescence of cells destined to undergo unbridled proliferation where E2F-1 knockdown Jurkat cells exhibited a significant increase in the translational profile of Mxi1 gene known to maintain the cell in the quiescent state [13, 21, 22]. The results reported here by means of reporter assay unambiguously reveal that it is through SREBP-1c response element present on the Mxi1 promoter sequence that E2F-1 can directly regulate Mxi1 expression (Fig. 9). Also ligand (Withaferin A) activated LXR-α known to induce SREBP-1c expression [8] can regulate Mxi1 expression level via SREBP-1c response

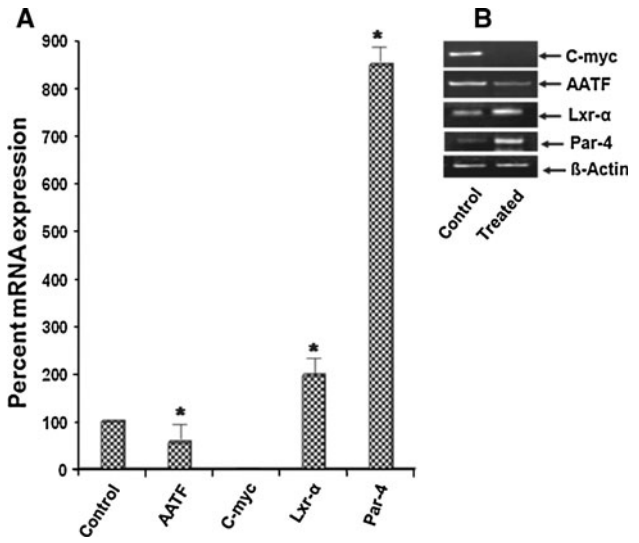

Fig. 14 **a** Transcriptional expression of AATF, c-myc, LXR-α, and Par-4 gene in control and Withaferin A treated Jurkat cells. mRNA expression of all the genes was normalized by β-actin mRNA expression. Each *bar* represents mean ± S.D. of the experiment done in triplicate; *statistical significance at $P < 0.05$. **b** Representative agarose gel photographs showing ethidium bromide stained RT-PCR products in control and Withaferin A treated Jurkat cells

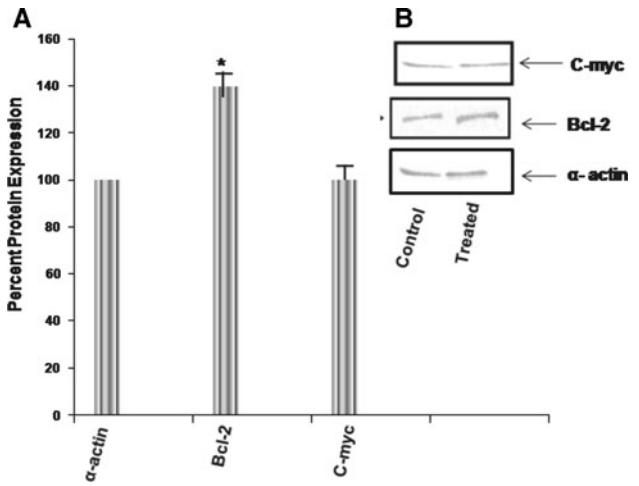

Fig. 15 **a** Translational expression of genes coding for Bcl-2 and c-myc in Withaferin A treated (10 μM) normal PBMCs. Protein expression of every gene was normalized by the expression of α-actin protein expression via western blotting. Each bar represents mean ± S.D. of the experiment done in triplicate; *statistical significance at $P < 0.05$. **b** Representative western blot showing translational expression of genes coding for Bcl-2 and c-myc in Withaferin A treated (10 μM) normal PBMCs

element present on its promoter sequence (Fig. 9). It is also confirmed from the reporter assay (Fig. 10) that Withaferin A effect on Leukemic cells apoptosis is through LXR-α. The observed huge amount of apoptosis as observed by FACS and also at ultrastructural level (Fig. 1, 5, 6, 7, 8) in the ligand activated LXR-α can be accounted to the fact that LXR-α activation leads to significantly high expression of Par-4 gene in the ligand treated when compared with untreated control Jurkat cells (Fig. 14) which in turn leads to a decreased translational profile of gene coding for Bcl-2, basically involved in inhibiting apoptotic process in a cell (Fig. 13). Further ligand activated LXR-α in Jurkat cells exhibited a dramatic loss of c-myc and E2F-1 gene expression at translational level (Fig. 13) followed by a significant decrease in the transcriptional expression of gene coding for AATF (Fig. 14) which is basically responsible for maintaining the proliferative potential of a cancerous cell [12]. The findings with LXR-α knockdown in Jurkat cells further strengthened the relationship which might exist between LXR-α biology and quiescence or apoptotic stages in cell cycle machinery. The results of this particular set of experiment actually mimicked opposite phenotypes as that obtained on LXR-α activation. Here the LXR-α knockdown Jurkat cells, taken as archetype cellular models, exhibited most of the cells in S phase/proliferative phase of cell cycle (Fig. 11) as compared to control Jurkat cells. Also the transcriptional profile of genes coding for c-myc and AATF showed a marked increase in the LXR-α knockdown cells as compared to control cells (Fig. 16) thus explaining the reason for an increased percentage of cells in the proliferative phase of cell cycle in this case as demonstrated by cell cycle analysis (Fig. 11). Hence, we propose that LXR-α gene may have the inherent ability to activate the genes responsible for quiescence (through SREBp-1c) as well as apoptotic processes of the cell cycle (Fig. 17). Also it is a well-known fact that LXR-α suppresses the master regulator NF-kappa B [7] which has the capacity to regulate many important genes involved in cell cycle so on the basis of these results, we propose a gene-regulatory pathway that might be responsible for regulating the cellular fate by the three important destinies of a cell, i.e., proliferation, apoptosis, and quiescence and also for devising a molecular link in relation to LXR-α with cholesterol homeostasis and inflammation (Fig. 17). Thus, oxysterol receptors in general and LXR-α in particular are well-suited targets for drug development as they are transcription factors easily activated/deactivated by small compounds that can penetrate the cell membrane and modulate receptor activity in vivo. LXR-α is an interesting drug target for pharmacological intervention of various metabolic disorders. The present and other recent reports establishing LXRs as regulators of cell growth indicate that LXR signaling may also be a potential target for anticancer drugs.

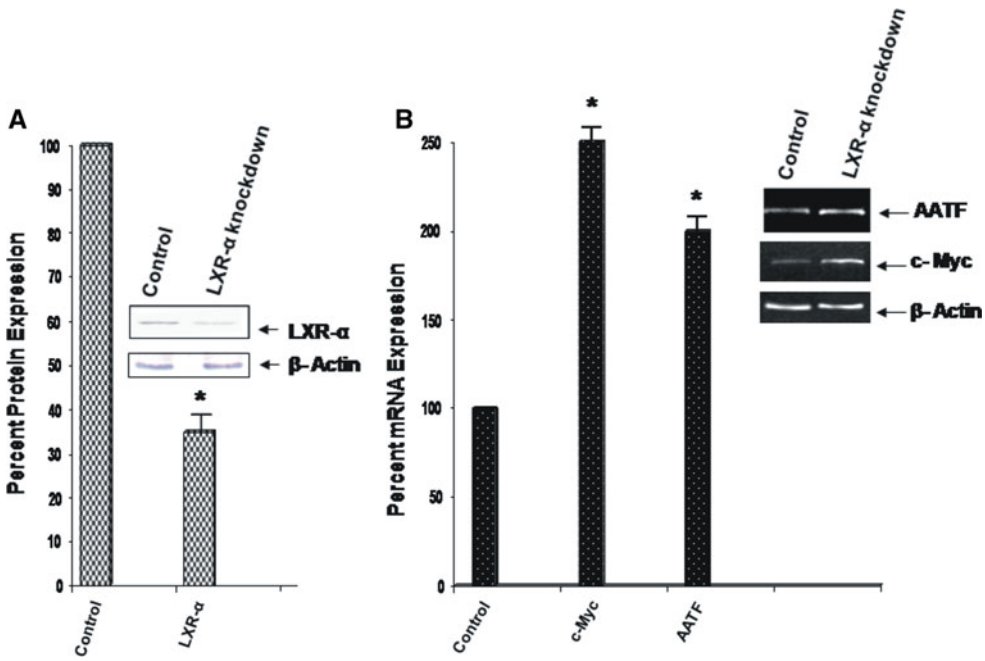

Fig. 16 **a** Representative western blot showing translational expression of gene coding for LXR-α in control and LXR-α knockdown Jurkat cells. *Bar* represents mean ± S.D. of the experiment done in triplicate; *statistical significance at $P < 0.05$. **b** Transcriptional expression of c-myc and AATF genes in control and LXR-α knockdown Jurkat cells. mRNA expression of c-myc and AATF were normalized by β-actin mRNA expression. Each *bar* represents mean ± S.D. of the experiment done in triplicate; *statistical significance at $P < 0.05$

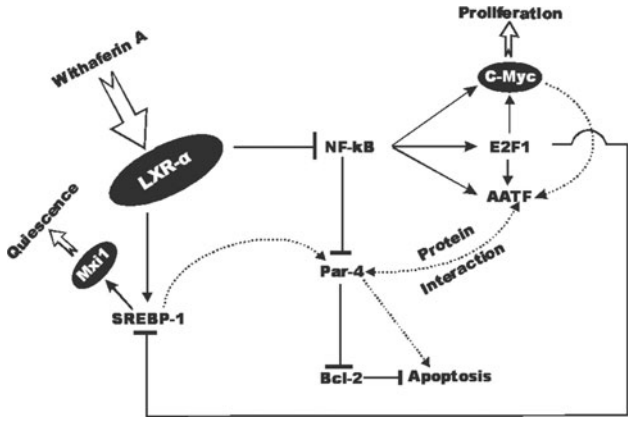

Fig. 17 Proposed LXR-α dependent gene-regulatory pathway that can induce T cell leukemic cells to enter into states characteristic of quiescence and apoptosis. LXR-α nuclear receptor/transcription factor regulates transcriptional expression of genes coding for NF-kappa B and SREBP-1c which in turn regulate the important genes involved in cell cycle regulation. This epigenetic phenomenon dictates the cancer cells specifically to decide to go for apoptosis and quiescence

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