RESEARCH ARTICLE

Molecular Target Therapy of AKT and NF-kB Signaling Pathways and Multidrug Resistance by Specific Cell Penetrating Inhibitor Peptides in HL-60 Cells

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Abstract

Background: PI3/AKT and NF-kB signaling pathways are constitutively active in acute myeloid leukemia and cross-talk between the two has been shown in various cancers. However, their role in acute myeloid leukemia has not been completely explored. We therefore used cell penetrating inhibitor peptides to define the contributions of AKT and NF-kB to survival and multi drug resistance (MDR) in HL-60 cells. Materials and Methods: Inhibition of AKT and NF-kB activity by AKT inhibitor peptide and NBD inhibitor peptide, respectively, resulted in decreased expression of mRNA for the MDR1 gene as assessed by real time PCR. In addition, treatment of HL-60 cells with AKT and NBD inhibitor peptides led to inhibition of cell viability and induction of apoptosis in a dose dependent manner as detected by flow cytometer. Results: Finally, co-treatment of HL-60 cells with sub-optimal doses of AKT and NBD inhibitor peptides led to synergistic apoptotic responses in AML cells. Conclusions: These data support a strong biological link between NF-kB and PI3-kinase/AKT pathways in the modulation of anti-apoptotic and multi drug resistant effects in AML cells. Synergistic targeting of these pathways using NF-kB and PI3-kinase/AK inhibitor peptides may have a therapeutic potential for AML and possibly other malignancies with constitutive activation of these pathways.

Keywords: AKT/PKB - NF-kB - cell penetrating peptides - AML - MDR - therapy

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Introduction

Acute myeloid leukemia account for about a third of all leukemia diagnosed in the world (Siegel, 2013). AML is characterized by an increased number of immature myeloid cells in bone marrow, which results in hematopoietic insufficiency (Lowenberg, 1999; Al-Bahar, 2008). A number of constitutively activated signaling pathways play critical roles in the survival and growth of Acute Myeloid Leukemia (AML) cells. These include PI3-kinase/AKT, MAP kinase, NF-kB and p53 pathways (Birkenkamp et al., 2004; Grandage, 2005). AKT/protein kinase B, a central component of the phosphoinositide3-kinase (PI3K) signaling pathways, is constitutively active in Acute Myeloid Leukemia cells (Grandage, 2005). PKB, a serine/threonine kinase, plays a key role in the regulation of numerous downstream targets, cellular anti-apoptosis, survival, cell growth and the cell cycle in many human cancers (Al-Bahar, 2008; Sophie, 2010; Grandage, 2005). AKT is composed of three conserved domains, an N-terminal pleckstrin homology (PH) domain, a central kinase catalytic (CAT) domain and a C-terminal extension (EXT) containing a regulatory hydrophobic motif (HM) (Kumar, 2005; Levitzki and Klein, 2010; Altman, 2011).

NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a family of transcription factors. The activation of NF-kB dimers is controlled by two major NF-kB signaling mechanisms referred to as the canonical (or classical) and non-canonical (or p100 processing) pathways. Phosphorylation of NEMO is an essential step in canonical NF-kB pathway activation (Thanos, 1995; Leith, 1997; Miyamoto, 2011). Inhibitors of NEMO has emerged as potential therapies against AML. This is because of, AML blasts have constitutive NF-kB activation (Furumai et al., 2001). The NF-kB survival pathway also has the ability to cross-talk with other survival pathways including PI3-kinase/AKT (Jui-Chuan Chuang 2012; M. G. KU Birkenkamp, H Schepers, J Westra, HH Lemmink, and E Vellenga, 2004).

AKT activation also NF-kB transcription factor

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induces drug resistance through MDR1 gene (Harris et al., 2013) expression in leukemic cells. Cellular mechanisms of drug resistance have been increasingly better defined for patients with acute leukemia and other hematologic malignancies. The best characterized resistance profile is the phenotype of multidrug-resistance (MDR) mediated by P-glycoprotein. P-glycoprotein is an ATP-dependent drug efflux pump for xenobiotic compounds with broad substrate specificity (Nørgaard, 2000). It is responsible for decreased drug accumulation in multidrug-resistant cells and often mediates the development of resistance to anticancer drugs (Weisburg, 2008; Talpaz, 2009). Therefore, targeting the AKT or NF-kB pathway alone may not be sufficient to induce apoptosis of cancer cells and combinations of various inhibitors maybe required to achieve the favorite effect. However, inhibitor these two pathways has not been elucidated in HL-60 cells.

The recent discovery of new potent therapeutic molecules which do not reach the clinic due to poor delivery and low bioavailability has made the delivery of molecules a keystone in therapeutic development. Peptide-based anticancer drugs have a potential to selectively target molecules and pathways deregulated in the trail of carcinogenesis (Chuang, 2012). Under these circumstances, the use of peptides, which copy 'natural' motifs that specifically influence kinase activity, may be a promising approach for selective inhibition of protein kinases (Eldar-Finkelman, 2009).Studies demonstrate that Cell Penetrating Peptide (CPP) transduction largely overcomes the problems associated with the more traditional transfection methods. Therefore, CPP-mediated transduction is generally non-toxic in the effective concentration ranges, it can rapidly delivered a diverse collection of molecular cargos into all cell types tested (Mayb, 2008). Natural and synthetic CPPs, are divided in three classes based on their biophysical properties: cationic (so named for the presence of arginine or lysine residues), hydrophobic, and amphipathic peptides (Tas, 2005).

In this study, we first determined whether inhibition of AKT and NF-kB activity by AKT inhibitor peptide and NBD (NEMO binding domain) inhibitor peptide, respectively, induce apoptosis in HL-60 cell lines. We also determined whether combined targeting of the NF-kB and the PI3-kinase/AKT pathways with sub-optimal doses of inhibitorswould induce a more potent apoptosis in HL-60 cells. Finally, in the present study, we report that the inhibition of AKT and NF-kB activity decrease MDR1 gene expression and drug resistance in HL-60 cells, significantly.

Materials and Methods

Cell culture, assessment of cell viability by trepan blue

The human AML, HL-60 cell linewas obtained from Pasteur Institute cell bank of Iran. HL-60 cells cultured in RPMI 1640 medium supplemented with 10% (v/v). Fetal bovine serum (FBS), 100 U/ml penicillin,100 U/ml streptomycin at 37˚C in an humidified at mosphere containing 5% CO_2. Viability of cells was determined by trepan blue assay. For implementation of MTT assay, % viability and number of cells were calculated as follow: % viability=viable cells/total cells ×100For MTT assay test we were used flasks with % viability >90%.

Reagents and peptides

AKT inhibitor VI, TAT-AKT-inhibitor peptide was purchased from EMB Millipore, USA (Cat. No.124013). AKT Inhibitor VI (AVTDHPDRLWAWEKF), a cell-permeable and reversible version of the AKT inhibitor peptide, fused with the protein transduction domain TAT (YGRKKRRQRRR) that displays antitumor properties. That inhibits the phosphorylation of AKT selectively and with minimal inhibition towards PKA, PKC, PDK1, p42/44 MAPK, or p38 MAPK. NBD inhibitor peptide was purchased from Gen Script, USA (Cat.No.RP20478). The NBD peptide (NEMO-binding domain peptide) is a cell-permeable synthetic peptide (TALDWSWLQTE), corresponding to the NEMO amino-terminal alpha helical region that has been fused to the antennapedia cell-permeable sequence (DRQIKIWFQNRRMKWKK) in the N-terminus, is shown to block TNF-alpha-induced NF-kB activation. The interaction of NEMO with the IKK complex is vital for the activation of the IKK complex and the subsequent activation of NF-kB. DMSO, Trypan blue and MTT were purchased from Sigma, Aldrich. YO-PRO®-1 stock solution and PI stock solution were purchased fromInvitrogen, USA. RNX-Plus solution was purchased from Cinnagen, Iran. First Strand cDNA Synthesis tube and GreenStar (2x) PCR master mixwere purchased from Bioneer,Korea. Primer was synthesis by Co. Takapozist, Iran.

Proliferation/death assays

Initially, HL-60 cells were seeded at the concentration of 3×10^4 cells in triplicates in a 96 well format. Cells were then treated with various doses of AKT inhibitor peptide and NBD (NEMO binding demine) inhibitor peptide in a final volume of 0.2 ml for 24, 48 and 72 hours. After incubation time, the cell culture medium was replaced with 200 µl fresh medium for 24 hours, after that the cells were incubated with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) solution for 4 hours. Then, contents of all wells were removed, 200 µl of pure DMSO were added to the wells followed by adding 25 µl Sorensen's glycine buffer to each well. The absorbance was read at 570 nm ELISA-reader. IC_{50} of drugs were measured by MTT assay, as previously described. The control cells were incubated with 20% of DMSO, and untreated cells. Each experiment was performed at least three times to confirm the results.

Assessment of apoptosis

To measure apoptosis,Vybrant® Apoptosis Assay Kitwas used as described previously. Briefly, 1×10^6 cells were treated with various sub-toxic doses of peptides alone or in combination for 48 hours. Harvest the cells after the incubation period, wash in cold phosphate-buffered saline (PBS) for each assay, and use a 1 mL assay volume. Add 1 µl YO-PRO®-1 stock solution and 1 µl Propidium Iodide stock solution to each reaction. Incubate the cells on ice for 20-30 minutes. As soon as possible after the incubation

period (within 1-2 hours), apoptotic cells were detected by flow cytometer analysis using a FACScan (Becton Dickinson, Mountain View, CA, USA) as described previously.

RNA extraction and real-time PCR

HL-60 cell lines were treated with different sub-toxic concentrations ofAKT, NBD inhibitor peptides alone or in combination for 48 hours. Total RNA was extracted from 1×10^6 cells using RNX-Plus solution. RNA concentrations were determined by Nano Drop (Eppendorf Bio Photometer) and at 260-280 nm purity of RNA wereassessed. The intactness of total RNA was confirmed by two sharp bands which are 28S rRNA and 18S rRNA separated on denaturing agarose geland visualized by DNA safe stained, under UV light (Figure 3). Reverse transcription was performedusing the First Strand cDNA Synthesis.Real-time PCR primers for beta-actin and multidrug-resistance 1 (MDR1) gens were purchased from Takapozist, IRAN. Primer sequences were as follows for MDR1 gene, 5'TCCATGCTCAGACAGGATGT3' (forward), 5'AACTTGAGCAGCATCATTGG3' reverse) and beta-actin gene (5'TGGACTTCGAGCAAGAGATG 3'(forward), 5'GAAGGAAGGCTGGAAGAGTG 3' (reverse). Relative quantitative real-time PCR used SYBR Green technology (Bioneer, Korea) on generated cDNAs. After pre-amplification 95°C, 10 min (Holding step), PCRs were amplified for 45 cycles; 95°C 15s (Denaturation); 60°C 45s (Annealing/Extension) on a Rotor Gene 6000 (Corbett). Each mRNA expression was normalized against beta-actin mRNA expression using the standard curve method.

Statistical analyses

Student's t test andANOVA test was performed to assess statistical significance among groups. Results with $p<0.05$ were considered statistically significant. Results were representedas mean±SEM of at least three independent experiments.

Results

MTT Assay

In this study we target HL-60 cell lines with AKT and NBD inhibitorpeptides alone or in combination for24,48 and 72h. MTT assay results show that the toxicity effect was increased by increasing the drugs dose, leading to the fact that these drugs are dose-dependent. This is quite the opposite for the viability factor. The AKT inhibitor and NBD inhibitor peptides shows with inhibitory concentration at 50 μM, 70 μM (IC_{50}), respectively, during 24, 48h and 72h. The sameness of results for different period's shows time-independency of this drug. The IC_{50} of the AKT and NBD inhibitor peptides were dose-dependent and it did not show significant time dependency (Figure 1).

Combination Treatment of HL-60 Cells with PI3-kinase/AKT inhibitor and NEMO inhibitor induce synergistically apoptosis

As shown in (Figure 2), AKT inhibitor peptide and NBD inhibitor peptide at a concentration of 5 μM and 7 μM could inhibit cell viability in HL-60 cell line. Cross-talk between NF-kB pathway and PI3-kinase/AKT pathway has been shown in various cancers. Utilizing this information; we aimed at targeting HL-60 cell lines with a combination of NF-kB and PI3AKT inhibitors at serial dilution of optimal doses to determine the synergistic therapeutic potential of such a combination. Multiple

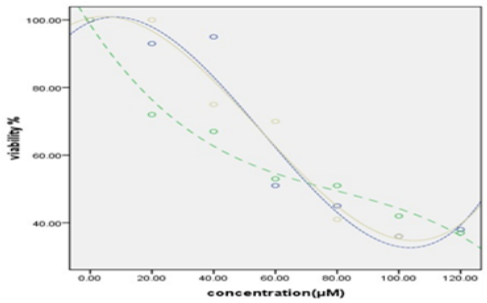

Figure 1. The NBD Inhibitor Peptide Shows with Inhibitory Concentration at 50% (IC_{50}), 70μM, in HL-60 Cell Line. IC_{50} was dose-dependent and it did not show significant time dependency, in 24, 48h and 72h

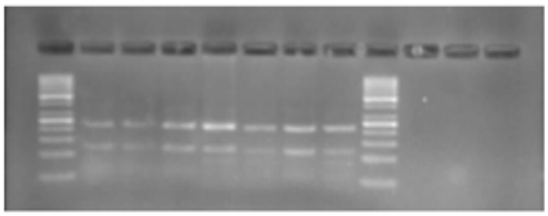

Figure 3. The Intactness of Total RNA was Confirmed by Two Sharp Bands which are 28S rRNA and 18S rRNA Separated on Denaturing Agarose Gels and Visualized by DNA Safe Stained, Under UV Light

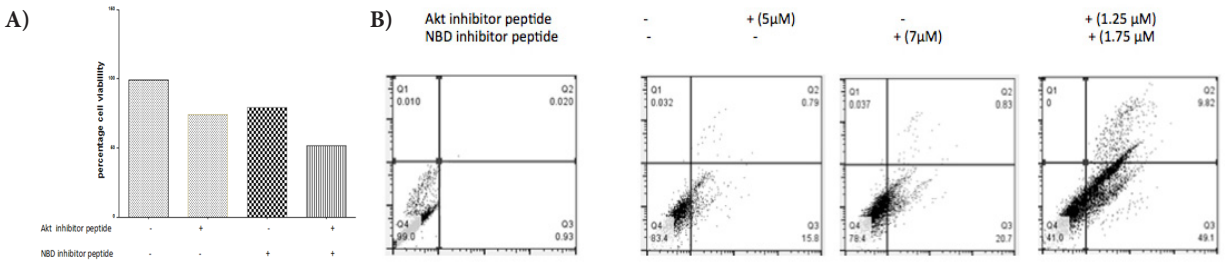

Figure 2. Combination of AKT and NF-kB Inhibitors Induce Synergistic Inhibition of Cell Viability and Induction of Apoptosis in HL-60 Cell Line. (A and B) Using of sub-toxic doses of AKT inhibitor and NBD inhibitor peptides suppresses growth of HL-60 cell. Combination of of sub-optimal doses of AKT inhibitor peptide 1.25 μM and NBD inhibitor peptide 1.75 μM , induces efficient apoptosis in HL-60 cells. cells were stained with FITC conjugated YO-PRO-1/PI and cells were analyzed by flow cytometry as described in material and methods

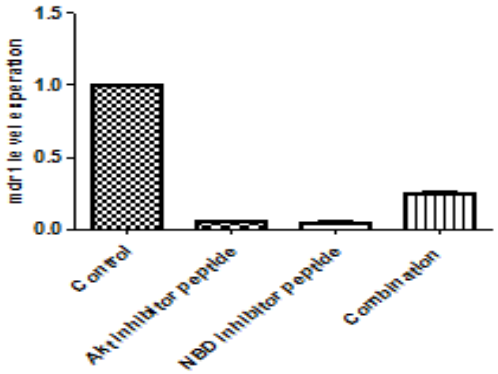

Figure 4. AKT Inhibitor Peptide and NBD Inhibitor Peptide at a Concentration of 5μM and 7μM, Respectively, Could Down Regulate MDR1 Gene Expression Up to 90% in HL-60 Cell

Table 1. Combination Index of AKT and NBD Inhibitor Peptides, Calculation using Chou and Talalay Method

AKT inhibitor peptide (μM)	NBD inhibitor peptide (μM)	Combination index (CI)
1.25	1.75	0.129
2.5	3.5	0.389
5	7	0.401
10	14	0.668
20	28	1.345

*1.25μM AKT inhibitor peptide and 1.75 μM NBD inhibitor peptide exerted the maximum synergistic response (combination index=0.129) with the values being less than 1.0 suggesting a strong synergistic response in HL-60 cell line

experiments were conducted to define optimal doses for a synergistic apoptotic response of the combination ofAKT inhibitor peptide and NBD inhibitor peptide. Combination of AKT inhibitor peptide and NBD inhibitor peptide, using Chou and Talalay method , 1.25μM AKT inhibitor peptide and 1.75 μM NBD inhibitor peptide exerted the maximum synergistic response in HL-60 cells (combination index=0.129) with the values being less than 1.0 suggesting a strong synergistic response, as shown in Table (1).

Quantitative mRNA analysis

The levels of MDR1 gene expression were measured by Real-Time PCR. Changes in MDR1 expression levels between the Control and treated HL-60 cells were normalized to beta-actin mRNA levels and then calculated by the $2^{-\Delta\Delta ct}$ method.AKT inhibitor peptide and NBD inhibitor peptide at a concentration of 5 μM and 7 μM could (4-5) fold down regulate MDR1 geneexpression in HL-60 cell. Gene expression can be reduced up to 95% (Figure 4).

Discussion

In this manuscript, we have hypothesized that a functional link might exist between AKT and NF-kB pathways in the pathogenesis of HL-60 cells and activation of these survival pathways may sustain survival and multi drug resistant of these malignant cells. Since, we proposed that simultaneous targeting of these pathways may synergistically induce apoptosis and anti- resistant role in HL-60 cells. We found that when HL-60 cell lines treated with sub-toxic doses, AKT inhibitor at a dose of 5 μM and NBD inhibitor at 7 μM could inhibit cell viability and MDR1gene expression in HL-60 cell lines. However when both the drugs were given together as a combination, there was efficient inhibition of cell viability (49%) of HL-60 cells in a sub-optimal doses of AKT inhibitor peptide and NBD inhibitor peptide 1.25 μM and 1.75 μM respectively. In addition, we found inhibitor peptide at a concentration of 5 μM and 7 μM could (4-5) fold down regulate MDR1 gene expression in HL-60 cell. Gene expression can be reduced up to 95%.

Acute myeloid leukemia (AML) is a very heterogeneous neoplasm of the hematopoietic stem cell (Sternberg, 2005). Despite important achievements in the treatment of AML, the long term survival of patients with the disease remains poor (Thol, 2011). A major goal for the development of new approaches for the treatment of AML is to design effective combinations targeting non-overlapping cellular pathways (Wu, 2006; Zeng, 2007; Seman, 2011). Selective inhibition of protein kinases is an extremely challenging goal of many drug discovery programs (Seman, 2011; Zeng, 2007). Based -peptide inhibitors have the advantage of selectivity due to their extensive interactions with the kinase-specific substrate binding site (Tal-Gan, 2011). The expression RNA of MDR1 and P-gp can be reduced through inhibiting NF-kappaB, so that the sensitivity of chemical therapy can be enhanced on hematologic malignant cells (Bentires-Alj, 2003; Wang, 2007).

These dataclearly indicate the importance of targeting multiple survival pathways simultaneously using sub-toxic doses of specific inhibitors thereby decreasing the chances of toxicity and increasing their response to therapy. Despite advances in therapeutic regimes for the treatment of aggressive AML over the last decade, AMLis still refractory to conventional systemic chemotherapy with a mean overall survivalof 5 years (Roberto, 2013). Therefore, newer therapeutic agents such as CCPs as a nanoparticles agent may play important roles in the management of these leukemia in combination with convention alchemotherapy to improve survival and decrease toxicity. In this study, we have investigated the anti-drug resistant role specific in hibitor of AKT and NF-kB pathways, using AKT inhibitor peptide and NBD inhibitor peptide in HL-60 cell lines.

In conclusion, our results demonstrate that the mechanism of AKT and NF-kB regulating anti-apoptosis has the correlation with the expression of MDR1geneand found that the NF-kB survival pathway also has the ability to cross-talk with AKT survival pathwaysin HL-60 cells.Targeting of AKT and NF-kB survival pathways simultaneously significantly increases the apoptotic stimuli and decrease drug resistant by suppress the MDR1 expression in HL-60 cells thereby decreasing the chances oftoxicity. Overall our data suggests that has the AKT inhibitor and NBD inhibitor cell Penetrating Peptides the rapeutic potentials against acute myeloid leukemia either alone or in combination with other inhibitors.

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