CDK inhibitors–induced SSAT expression requires NFκB and PPARγ in MCF-7 breast cancer cells

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Abstract: The cyclin-dependent kinase (CDK) inhibitors purvalanol and roscovitine are therapeutic agents that control cell proliferation through regulating cell-cycle machinery. They also affect polyamine (PA) metabolism, which is activated in malignant tissues. Therefore, PA catabolism became a remarkable target in cancer therapies. Induction of the PA catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) is under the control of transcription factors such as NFκB and PPARγ. The purpose of this study was to investigate the therapeutic potential of CDK inhibitors in combination with PAs in MCF-7 breast cancer cells. In order to understand the involvement of PA catabolic enzyme SSAT in this process we also checked its transcriptional regulation in the presence of CDK inhibitors. MCF-7 cells were exposed to CDK inhibitors in the absence or presence of Spd and Spm. Cell viability loss was evaluated by MTT assay. Apoptosis was determined by annexin-V/PI staining using FACS flow. The SSAT transcription level was measured by qRT-PCR. Intracellular PA pool was determined by HPLC. Protein expressions were assessed by western blotting. We found that CDK inhibitors decreased cell viability in a time-dependent manner and induced apoptosis. Co-treatment of Spd or Spm with CDK inhibitors prevented the apoptotic potential of both drugs. Purvalanol increased SSAT expression levels in a time-dependent manner. Although the induction of SSAT by purvalanol resulted in the activation of NFκB at early time points, induction was accomplished by PPARγ as a late response after purvalanol treatment. We concluded that both transcriptional control mechanisms could be responsible for SSAT regulation in a time-dependent manner.

Key words: Polyamines, SSAT, purvalanol, PPARγ, NFκB

1. Introduction

The interaction of cyclin-dependent kinases (CDK) and their specific cyclin counterparts regulates cell-cycle progression. Deregulation of the cell cycle induces the transformation of normal cells into malignant cells. The CDK inhibitors purine-derived purvalanol or roscovitine (ROSC, CYC202) block the ATP binding sites of CDK1, CDK2, CDK5, and CDK7 and cause cell-cycle arrest at G1/S or G2/M, which leads to apoptosis in various cancer cell lines.

The polyamines (PAs) putrescine (Put), spermidine (Spd), and spermine (Spm) are essential amine derivatives with cationic properties and have important roles in cell growth and differentiation. PA levels are found at higher concentrations in malignant tissues than in normal tissues (Simoneau et al., 2001), which indicates the potential of these molecules as targets for antineoplastic therapy. PA catabolism is under the control of spermidine/spermine

N^1-acetyltransferase (SSAT) and polyamine oxidase (PAO). While SSAT establishes the first step of catabolic activity by acetylating Spd or Spm, it also prepares these molecules to be excreted from the cell. SSAT activity is also required to present substrates for PAO, which oxidizes acetylated forms as the second step of PA catabolism. The byproducts of these reactions are H_2O_2 and 3-acetoaminopropanol. SSAT is upregulated by various chemotherapeutic agents in several tumor types (Casero et al., 2003; Murray-Stewart et al., 2003; Zahedi et al., 2003). CDK inhibitors could trigger apoptotic machinery by activating SSAT and related reactive oxygen species in different cancer cell lines such as colon and prostate (Arisan et al., 2012; Coker et al., 2013; Obakan et al., 2013; Obakan et al., 2014). Recent studies suggest that Spd and Spm exert multiple effects including antioxidant and antiinflammatory responses. While Spd treatment prolonged the life span of several model organisms by preventing the protein damage that

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caused oxidative aggregation (Eisenberg et al., 2009), Spm might promote drug-induced apoptosis or sensitivity to therapy models in cancer cells (Agostinelli et al., 2007). In primary human cells, Spm treatment resulted in an intracellular increase of reactive oxygen species (ROS) and apoptosis triggered via mitochondria membrane potential loss (Schiller et al., 2005). However, Spm-mediated cell survival was detected in neuron cells in a concentration-dependent manner (Chu et al., 1994).

The transcriptional regulation of SSAT is under the control of PAs, which might also affect other important transcription factors: the nuclear factor kappa-light-chain enhancer of activated B-cells (NFκB) and peroxisome-proliferator–activated receptor gamma (PPARγ). NFκB plays crucial roles in the development and progression of cancer. It regulates more than 400 genes involved in cell survival, cell proliferation, invasion, angiogenesis, and metastasis (Sethi et al., 2008; Yadav et al., 2010). Homo- or heterodimers of the Rel family proteins, p50/NF-κB1, p52/NF-κB2, p65/RelA, and c-Rel are the main molecules to form NFκB dimers (Karakas and Gozuacik, 2014). Under normal conditions, NFκB dimers are located in the cytoplasm through a physical association with IκBα in most cell types (Baeuerle et al., 1988). When the cells are stimulated by tumor necrosis factor (TNF), lipopolysaccharide (LPS), interleukin-1 (IL-1), or chemokine (CD40) signals under stress conditions, IκBα becomes hyperphosphorylated by IκBα kinase (IKK) on distinct serine residues; this hyperphosphorylation induces the proteolytic degradation of IκBα. The degradation of IκBα eventually leads to the dissociation of NFκB dimers, allowing its translocation to the nucleus, where it binds with high specificity to the enhancer sequences in the promotors of target genes. PPARγ belongs to the nuclear hormone receptor superfamily and plays an essential role in the regulation of cellular differentiation, development, metabolism, and tumorigenesis. Two PPARγ response elements in the promoter of the SSAT gene were identified (Babbar et al., 2003).

Our aim was to investigate the possible role of CDK inhibitors on the SSAT expression profile by examining the modulation of NFkB or PPARγ in estrogen-positive MCF-7 breast cancer cells. We found that purvalanol could activate SSAT expression which led to a decrease in total intracellular PA content. The induction of SSAT by purvalanol could be accomplished by PPARγ as a late response, whereas p65 was responsible for SSAT upregulation as an early response (within 4 h). The upstream regulatory elements of NFκB, IκKα, and IκBα were activated by purvalanol treatment in MCF-7 cells. As a result of a similar modulation of PPARγ in the presence of purvalanol we concluded that both transcriptional control mechanisms could be responsible for SSAT regulation in a time-dependent manner.

2. Materials and methods

2.1. Cell lines
MCF-7 breast cancer cells (ATCC) were grown in DMEM medium (PAN Biotech) supplemented with 10% fetal bovine serum (PAN Biotech) and penicillin-streptomycin (10,000 U penicillin/mL; 10 mg streptomycin/mL) at 37°C in a humidified 5% CO_2 incubator. Cells were seeded overnight and then treated with purvalanol (Tocris Bioscience, Bristol, UK), 10 mM stock concentration in DMSO and stored at –20 °C, or roscovitine (Calbiochem, La Jolla, CA, USA), 10 mM stock concentration in DMSO, and N-acetylcysteine (NAC) (Sigma Aldrich, St Louis, MO, USA). The effect of serum amine oxidases as a possible source of ROS was excluded by their inhibitor, aminoguanidine (Sigma Aldrich, St Louis, MO, USA).

2.2. Cell viability assay
The effect of CDK inhibitors on cell viability was determined by a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Roche, Indianapolis, IN, USA). MCF-7 cells were plated at 1×10^5 cells/well in 96-well plates, allowed to attach overnight, and treated for 24 h with various concentrations of purvalanol or roscovitine (20 and 25 μM, respectively). After a 24 h treatment, 10 μL of MTT (5 mg/mL) was added to the culture medium and incubated for 4 h. Following aspiration of the medium, 200 μL of DMSO was added to dissolve the formazan crystals. The absorbance of the suspension at 570 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

2.3. Flow cytometric analysis of apoptosis
Cells (2×10^5 cells/well) were seeded in 6-well plates and then treated with purvalanol and roscovitine for 24 h (20 and 25 μM, respectively). Cells were collected and washed twice with cold 1X PBS. The cell pellet was resuspended in 300 μL of binding buffer and incubated with 3 μL of FITC-conjugated annexin-V and 3 μL of PI (BD Biosciences, San Jose, CA, USA) for 15 min at room temperature in the dark at 37 °C in a humidified 5% CO_2 incubator. The samples were analyzed by flow cytometry (Accuri Cytometers, Inc., Ann Arbor, MI, USA)

2.4. Determination of reactive oxygen species (ROS) by DCFH-DA staining
MCF-7 cells were seeded in 6-well plates (1×10^5 cells/well). Following exposure of cells to purvalanol or roscovitine for 24 h, media was carefully discarded. Cells were trypsinized, resuspended in 1X PBS, and stained with DCFH-DA (0.5 μM) (Molecular Probes, Inc., Eugene, OR, USA) for 45 min in a 5% CO_2 incubator. After the incubation period we added propidium iodide 0.5 μL/0.5 mL/tube (final concentration = 1 μg/mL) and proceeded to flow cytometry analysis (Attune, Applied Biosystems). Living cells, which are PI negative, were selected by FACS gating. In these living cells we recorded the fluorescence of DCF on the FL-1 channel (525 nm).

2.5. RNA extraction and qRT-PCR

Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. cDNAs from each samples were obtained by using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The cDNAs were subjected to quantitative real-time PCR. Forty cycles of reactions were performed under the conditions of denaturation for 30 s at 95 °C; annealing was performed for 30 s at 55 °C and extension for 30 s at 72 °C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. PCRs were performed in separate tubes in duplicate in a MiniOpticon (Bio-Rad, Hercules, CA, USA). SSAT cDNAs were amplified using SSAT primers purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GAPDH internal standard cDNA was also amplified using the following primers:

forward,5'-CGGAGTCAACGGATTTGGTCGTAT-3';
reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC -3'.

2.6. Western blot analysis

For total protein extraction from the MCF-7 cell line, cells were lysed in ProteoJET mammalian cell lysis reagent (Fermentas, Hanover, MD, USA) containing total protease inhibitor cocktail (Fermentas, Hanover, MD, USA). Total cellular protein level was determined by the Bradford method (Bio-Rad). For cytoplasmic and nuclear protein extraction a NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Scientific, Rockford, IL, USA) was used, according to manufacturer's instructions.

To proceed to immunoblotting, 20 μg of total protein was loaded onto 12% SDS-polyacrylamide gels and subjected to electrophoresis. Gels were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Mannheim, Germany). PVDF membranes were rinsed in tris buffer saline with Tween 20 (TBS-T) (10 mM Tris-HCl, pH 8; 0.05% Tween 20) and blocked in a buffer of TBS-T containing 5% skim milk overnight at 4 °C. PVDF membranes were then incubated with primary antibodies (SSAT, p65, IκBα, P-IκBα, P-IKKα, P-p65, and PPARγ; β-tubulin used as loading control) (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. Membranes were then incubated with appropriate secondary antibodies (anti-rabbit or anti-mouse IgG) conjugated to horseradish peroxidase for 2 h at room temperature. Membranes were developed using ECL chemiluminescence reagents (Amersham Biosciences, Princeton, NJ, USA) and exposed to X-ray films (Kodak).

2.7. Measurement of polyamine levels

Cells (3×10^5) were harvested from 6-well culture petri dishes, washed twice with PBS, and pelletized. The cell pellet was treated with 50% tricholoroacetic acid and centrifuged at 13,200 rpm for 20 min. Supernatant was retained, and benzoylation was performed. PA levels were determined by HPLC (Agilent Technologies, Santa Clara, CA, USA), as described by Singh et al. (1989).

2.8. Statistics

Numerical data were obtained from the averages of at least two experiments and analyzed with Graph Pad version 4.04 software. To determine significant alterations, a two-tailed unpaired t-test was performed. Immunoblotting results were repeated at least twice, and ImageJ software was used to measure the band intensities.

3. Results

3.1. Co-treatment of Spd and Spm with each CDK inhibitor altered the cytotoxic effect

To understand the effect of the potential role of PAs on the cytotoxic effect of purvalanol and roscovitine, a MTT cell viability assay was performed in MCF-7 cells. As shown in Figure 1a, purvalanol (20 μM) and roscovitine (25 μM) decreased cell viability by 40% and 50%, respectively. Spd and Spm co-treatment at a 10 μM concentration for 24 h prevented CDK inhibitor-induced cell viability loss (Figure 1a).

In order to understand the effects of Spd or Spm co-treatments on CDK inhibitor-induced apoptosis, we performed the annexin-V/PI staining and analyzed apoptotic populations with a flow cytometer. As shown in Figure 1b, early and late apoptotic population percentages increased following purvalanol and roscovitine treatments by 16% and 12%, respectively, compared to untreated control samples. However, while Spd and Spm did not cause apoptotic cell death, as compared to control samples, combined treatments with either Spd or Spm significantly decreased drug-induced apoptosis (Figure 1b). Spd co-treatment prevented apoptotic effects of purvalanol and roscovitine by 13% and 12%, respectively. These rates were 17% and 10% when MCF-7 cells were treated with each CDK inhibitor in the presence of Spm.

3.2. Co-treatment of polyamines prevented ROS generation

Generation of ROS, an apoptosis-inducing factor in cancer cells, is generally correlated with the active PA catabolic machinery. We found that ROS generation was triggered after CDK inhibitor treatment, and additional polyamine treatment prevented drug-induced accumulation of ROS in MCF-7 cells (Figure 2a). Intracellular ROS accumulation was detected with DCFH-DA staining following drug treatment for 24 h. Co-treatment of cells with either Spd or Spm prevented ROS accumulation induced by purvalanol and roscovitine, which supports the suggestion that exogenous PAs might withdraw the apoptotic effect of CDK inhibitors via ROS accumulation in MCF-7 cells. As shown in Figure 2b, the antioxidant
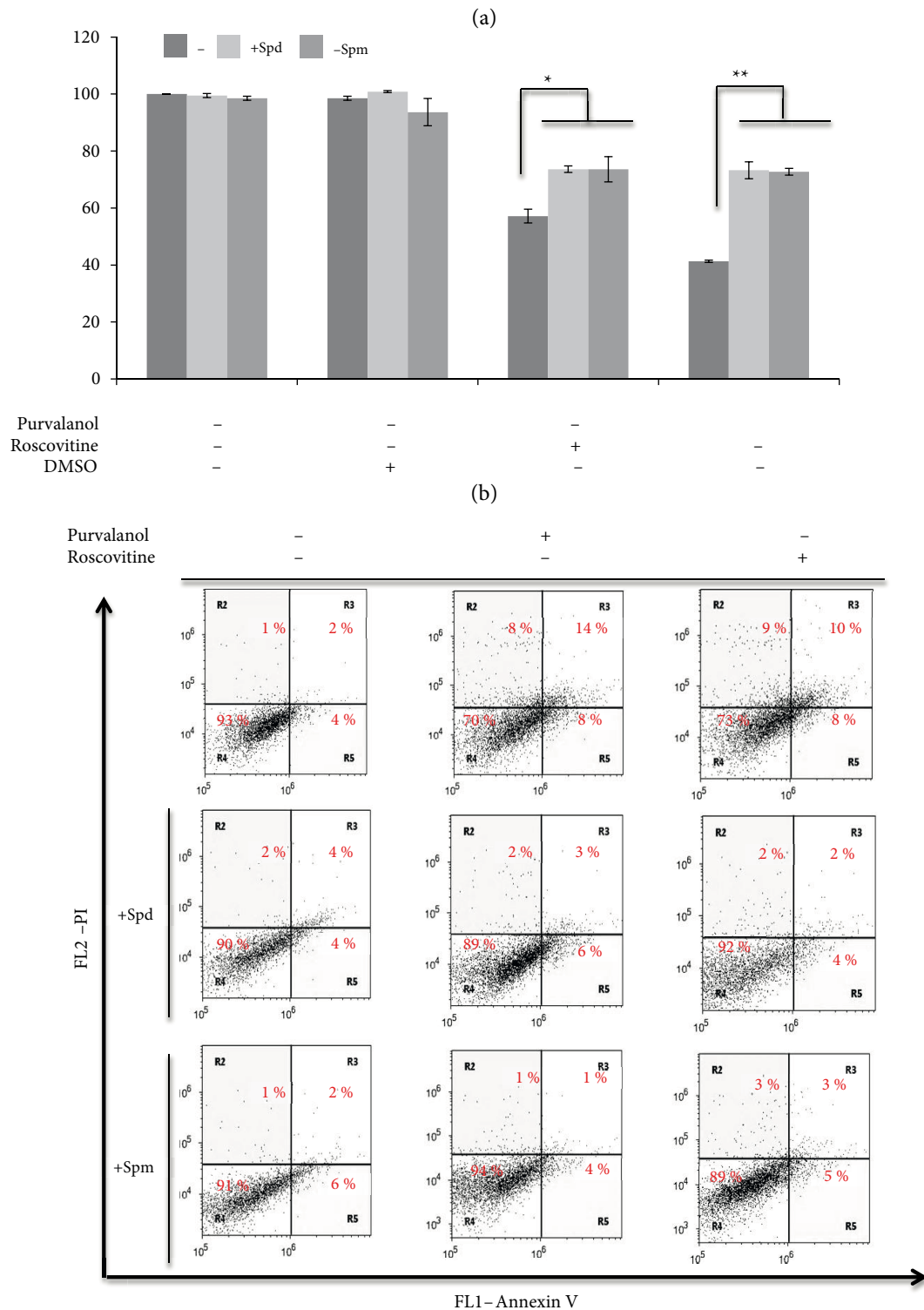
Figure 1. Purvalanol and roscovitine induce cell death in MCF-7 breast cancer cells. a. Cells (1×10^4) were seeded into 96-well plate and treated with purvalanol and roscovitine, either in the presence of Spm or Spd, for 24 h. MTT cell viability assay was performed. The result shown is the average from three independent trials with at least four replicates. Statistical differences were analyzed using an unpaired t-test; *P < 0.05. b. Apoptosis was evaluated with Annexin-V and PI staining following purvalanol and roscovitine treatment by flow cytometry. Annexin-V FITC staining in x-axis and PI in y-axis. The number represents the percentage of healthy (lower-left), early apoptotic (lower-right), late apoptotic (upper-right), and necrotic (upper-left) populations.
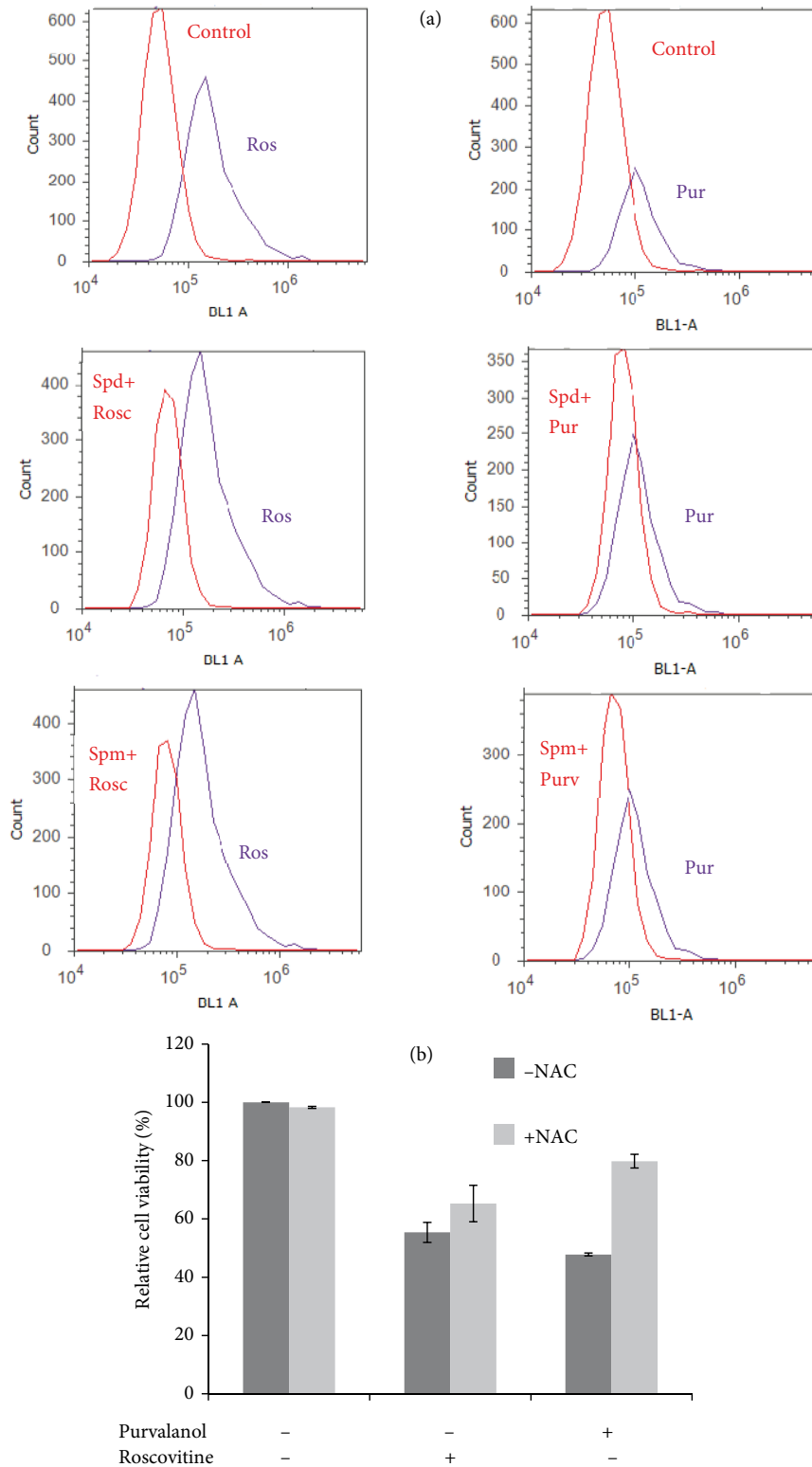
Figure 2. Co-treatment of CDK inhibitors with PAs prevented reactive oxygen species (ROS) generation. a. MCF-7 cells were seeded in a 96-well plate (1×10^4 cell/well) and treated with purvalanol and roscovitine, with or without Spd and Spm, for 24 h. Following DCFDH-DA (1 µg/mL) staining for 30 min, ROS generation was determined by flow cytometry. b. MCF-7 cells co-treated with CDK inhibitors and 5 µM NAC. The MTT assay was performed to determine cell viability. The result shown is the average from three independent trials with at least four replicates.

N-acetylcysteine (NAC) protected MCF-7 cells against purvalanol- and roscovitine-induced cell death by 30% and 10%, respectively.

3.3. Depletion of intracellular polyamine pool was correlated with SSAT induction

Since clarification of activated PA catabolic machinery might induce apoptosis due to the generation of hydrogen peroxide and aldehydes in response to chemotherapeutic agents, we checked the time-dependent effect of purvalanol on the expression status of SSAT, the first-step enzyme of PA catabolism by qRT-PCR method. As shown in Figure 3a, purvalanol treatment increased SSAT expression levels within 8 h. Prolonged exposure to purvalanol caused a fluctuation in gene expression within 24 h. According to
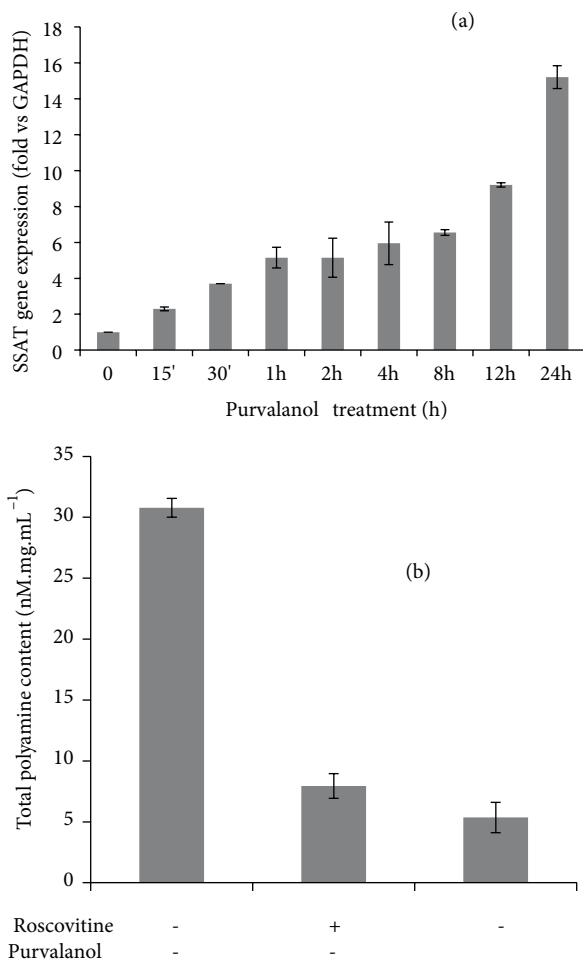
qRT-PCR results, SSAT expression is mostly upregulated within 12 h. Finally, as shown in Figure 3b, we also determined the intracellular PA pool by HPLC. Both CDK inhibitors depleted PA pool in MCF-7 cells.

3.4. NFκB and its upstream enzymes were downregulated in response to purvalanol

The phosphorylation of IKKα, the upstream kinase of IκBα, was also investigated to evaluate the potential role of NFκB signaling in purvalanol-modulated polyamine metabolism after CDK inhibitor treatment. Purvalanol treatment caused dephosphorylation of p-IKKα, as well as p-IκBα, within 24 h. Purvalanol was effective at dephosphorylating p65 at Ser536 residue. However, roscovitine exerted a lesser effect on the NFκB-signaling pathway (Figure 4a). Therefore, we concluded that purvalanol modulated NFκB signaling more effectively than roscovitine treatment in MCF-7 breast cancer cells. These results were confirmed by presentation of total protein expression levels of NFκB p65 subunit and its regulatory upstream element IκBα, which was altered in a time-dependent manner following purvalanol treatment (Figure 4b). Exposing cells to purvalanol decreased p65 expression within 2 h, but it was restored following 4- and 12-h treatments. We also checked the total expression level of PPARg, which is one of the responsible transcription factors for SSAT, following purvalanol treatment (Figure 4b). Purvalanol treatment starting from 15 min upregulated the PPARg expression level within 24 h. The nuclear translocation of p65 following purvalanol treatment was shown by isolating nuclear/cytoplasmic protein lysates in MCF-7 cells. As shown in Figure 4c, translocation of p65 to the nucleus was prevented after a 24-h purvalanol treatment. As p65 translocation was prevented and polyamine levels decreased with 24 h purvalanol treatment through induction of SSAT, we investigated the other SSAT-inducing transcription factor, PPARγ. As shown in Figure 4c, translocation of PPARγ to the nucleus was more effectively induced by purvalanol than roscovitine in MCF-7 cells.

4. Discussion

The correct functional status of CDKs is required for the achievement of the cell cycle. Therefore researchers are focused on CDK-targeted therapies to block cell-cycle progress to induce apoptosis in cancer cells. Purvalanol and roscovitine are synthetic CDK inhibitors used in the treatment of breast, prostate, and colon carcinomas (Wesierska-Gadek et al., 2004; Arisan et al., 2011). Roscovitine is the most studied CDK inhibitor and was a strong apoptotic inducer in various cell lines. It exerts its therapeutic effect by targeting cell cycle arrest at the G1/S or G2/M phase transition points due to inhibition of CDK1, CDK2, and CDK5 in lung and cervical cancer cells. Although similar mechanisms are activated by purvalanol,
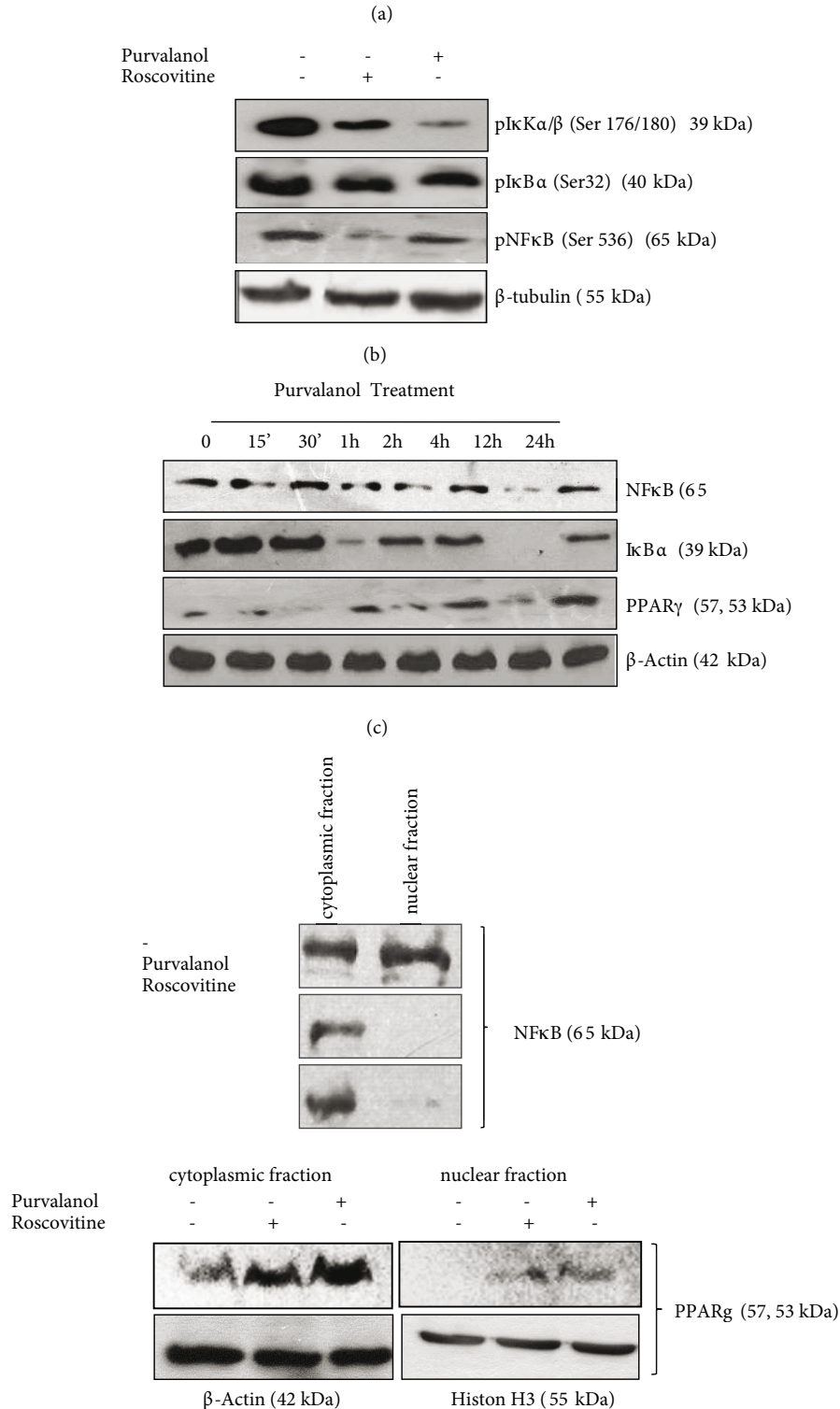

Figure 3. Purvalanol induced SSAT gene expression in MCF-7 cells. a. MCF-7 cells were treated with purvalanol in a time-dependent manner and harvested, and total RNA was extracted and used for qRT-PCR. SSAT expression was normalized to GAPDH expression. Fold induction was calculated by dividing normalized values of the sample by the control value. The result was shown as the mean ± SD from three different experiments. b. The effect of roscovitine or purvalanol on intracellular polyamine levels was analyzed by HPLC.

(a)

(b)

Purvalanol Treatment

(c)

Figure 4. Purvalanol and roscovitine suppress the NFκB signaling pathway and induce PPARγ. a. Expression profiles of pIKKα, IkBα, and pNFκB were determined by immunoblotting after purvalanol treatment. b. Expression profiles of NFκB, IκBα, and PPARγ were determined by immunoblotting following time-dependent purvalanol treatment in MCF-7 cells. β-Tubulin was used as a loading control. c. Nuclear translocation of NFκB and PPARγ were determined by immunoblotting. β-Actin and histone H3 were used as loading controls for cytoplasmic and nuclear fractions, respectively.

less information is available to evaluate its potential therapeutic effect in cancer cells. Previous studies showed that purvalanol caused downregulation of Rb, cyclin E, and cyclin A to block the cell cycle in mouse fibroblast BP-A31 cells (Villerbu et al., 2002). In addition, nuclear fragmentation due to purvalanol treatment was shown in HT-29 cells (Villerbu et al., 2002). Similar to these results, we found that both CDK inhibitors significantly decreased cell viability compared to untreated cells within 24 h in MCF-7 breast cancer cells (Figure 1a). Additional Spd or Spm treatment prevented drug-induced cytotoxicity and apoptosis (Figure 1b).

PAs are essential polycationic compounds that have critical roles to regulate transcription, translation, and proliferation in cellular homeostasis. The increase in total PA levels was detected in malignant cells, and this promotes cell proliferation. Therefore, PAs are suggested as attractive targets for chemotherapeutic interventions. Intracellular PA levels are involved in the regulation of cell proliferation in different ways depending on their concentration and metabolism. Spd and Spm are polycationic molecules, and they stabilize the DNA/RNA (Obakan et al., 2014). High intracellular levels of Spm induce production of oxidative molecules that increase DNA damage and cell death; either apoptosis or necrosis. In the present study, exposure of cells to Spd and Spm protected cells from CDK inhibitor-induced cell viability loss and apoptosis (Figures 1a and 1b). Our results were correlated with the finding that co-treatment with Spd protected Ehrlich ascites tumor cells from apoptosis induced by 1'-acetoxychavicol acetate (Moffatt et al., 2000). In addition, similar to our data, Spd treatment has been shown to suppress ageing on yeast, flies, and worms (Eisenberg et al., 2009). Conversely, Spm treatment triggered apoptosis via induction of oxidative stress in a variety of cell types, including cardiac myoblasts (Maccarone et al., 2001; Hirano et al., 2006). PA catabolic enzyme overexpression is known with apoptosis-promoting roles due to generation of the toxic byproducts hydrogen peroxide and aldehydes in response to chemotherapeutic agents. In our previous studies, we showed that CDK inhibitors are responsible for inducing PA catabolic enzymes, PAO, and SSAT. As a response to CDK inhibitor-induced PA catabolic enzyme levels, ROS generation could be related to induction of SSAT. Previous studies reported ROS generation is linked with DNA damage and results in apoptosis (Casero et al., 2003; Arisan et al., 2012). Our previous studies showed that purvalanol and roscovitine induce ROS generation in LNCaP, DU145, PC3 prostate cancer cells, and HCT116 colon cancer cells (Arisan et al., 2012; Coker et al., 2013; Obakan et al., 2014). Spm co-treated cells showed a greater increase in DCFDH-DA staining than Spd co-treatment with CDK inhibitor. In contrast to our results in MCF-7, when TNFα

combined with Spm, apoptosis was blocked due to the free radical scavenger role of Spm (Naredi et al., 1994). Our observation that N-acetylcysteine (NAC) protected MCF-7 breast cancer cells from CDK inhibitor-induced apoptosis by inhibiting ROS accumulation also confirmed that both CDK inhibitors could act by inducing ROS generation. Similar to our results, in chemotherapeutic agents such as docosahexaenoic acid apoptosis was triggered via ROS generation, which could be inhibited with the NAC in MCF-7 cells

In our previous work, we showed that purvalanol and roscovitine induced SSAT expression and decreased the intracellular polyamine levels in HCT116 colon adenocarcinoma cells (Arisan et al., 2011). Because polyamines are essential for malignant cell growth, in our present paper we evaluated the effects of purvalanol on PA catabolism and determined the mechanism by which purvalanol was able to induce SSAT expression in a time-dependent manner in MCF-7 cells. The induction of SSAT is usually accompanied by a decrease in intracellular levels of PA, in particular Spd and Spm. Our results indicated that purvalanol at 20 µM led to a decrease in total PA levels after 24 h. Recent reports suggest that many chemotherapeutic drugs such as polyamine analogues, BENSpm (Huang et al., 2005), roscovitine (Coker et al., 2012), and sulindac (Babbar et al., 2003) lead to the induction of SSAT and a lower intracellular PA pool in MCF-7 and other cancer cells.

The induction of SSAT due to purvalanol treatment led us to investigate the involvement of SSAT transcription factors, NKκB and PPARγ. The SSAT promoter sequence has several potential responsive binding sites, -286, -594, and -1735; three NKκB sites (Babbar et al., 2006). Aspirin activates SSAT transcription via two NFκB response elements (NRE) in the SSAT 5' promoter flanking sequence (Babbar et al., 2006). NKκB is formed by homo- and hetero-dimers of p50 and p65 proteins in the cytoplasm. They have been shown to play a role in tumor development and control the expression of genes involved in cell-cycle regulation and apoptosis (Basseres et al., 2006). For this reason, we first investigated the NFκB regulatory proteins, phosphorylated IKKβ, and the downstream target IκBα phosphorylation. Phosphorylation of IκB is required for its ubiquitination and proteasomal degradation by allowing the release and activation of NFκB. In our experimental system, purvalanol treatment decreased IKKβ phosphorylation, and it could not phosphorylate IκBα more significantly than roscovitine treatment. This result suggested that the classical NKκB pathway for translocation is blocked by CDK inhibitors. In addition, both drugs dephosphorylated NKκB complex member p65, which indicated that CDK inhibitors prevented the translocation of NKκB to the nucleus

after 24 h. It is well established that Spm treatment could activate estrogen receptor (ER)-α and NFκB-mediated transcription. Electromobility shift assay (EMSA) and immunoprecipitation studies demonstrated that Spm enhanced ERα-estrogen response element (ERE) and NRE binding, which cause increased nuclear levels of NFκB and provided stronger binding efficiency to DNA and protein components for transcriptional initiation (Shah et al., 2001). Similar to our results, epidermal growth factor inhibitor celecoxib inhibited the translocation of NFκB in A459, GLC82, and SW 1573 lung cancer cells (Chen et al., 2008). BMS-345541 also blocks NFκB activation by inhibition of IKKα in HTLV-1 (Agbottah et al., 2008). The time-dependent expression profile of NKκB and IκBα also showed that these proteins were downregulated following purvalanol treatment in a time-dependent manner. However, our results from qRT-PCR for SSAT showed transcriptional induction reached the highest level in 24 h; we therefore investigated potential SSAT transcription activators other than NKκB. We first determined the total PPARγ expression level following purvalanol treatment in a time-dependent manner. We found that total PPARγ expression levels increased compared to untreated MCF-7 cells in a fluctuating manner; however, 12 and 24 h seemed critical because at these same time periods NKκB expression downregulated, although SSAT expression continued to increase. Therefore, we determined the translocation profile of each transcription factor to the nucleus. Either 24 h purvalanol or roscovitine treatment totally inhibited the translocation of NKκB to the nucleus. In addition, the cytoplasmic levels of NFκB decreased after purvalanol treatment for 24 h. Furthermore, purvalanol and roscovitine induced the translocation of PPARγ, as well the cytoplasmic PPARγ, to the nucleus. PPARγ induction is targeted to counteract hyperglycemia (Atanasov et al., 2013). Interestingly, purvalanol has been shown to inhibit NKκB and trigger apoptosis in human T-cell leukemia virus type-1 (HTVL-1) (Agbottah et al., 2008). Therefore, we conclude that although purvalanol could induce the transcription of SSAT under control of NFκB as an early response, NFκB expression begins to downregulate after prolonged treatment with purvalanol in order to switch off the NFκB-dependent survival signal. However, cells could continue to express SSAT, and generate ROS and apoptosis, after long-term purvalanol treatment under control of PPARγ.

5. Conclusion

Our data demonstrated that there is a close relationship between PPARγ and NFκB in SSAT transcription following purvalanol treatment in MCF-7 cells. The purvalanol exposure time is the critical factor for the induction of SSAT expression in MCF-7 breast cancer cells.

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