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ORIGINAL ARTICLE

Polyamine depletion enhances the roscovitine-induced apoptosis through the activation of mitochondria in HCT116 colon carcinoma cells

Elif Damla Arısan · Ajda Çoker · Narçin Palavan-Ünsal

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Abstract Small molecule inhibitors of cyclin-dependent kinases (CDKs) show high therapeutic potential in various cancer types which are characterized by the accumulation of transformed cells due to impaired apoptotic machinery. Roscovitine, a CDK inhibitor showed to be a potent apoptotic inducer in several cancer cells. Polyamines, putrescine, spermidine and spermine, are biogenic amines involved in many cellular processes, including apoptosis. In this study, we explored the potential role of polyamines in roscovitine-induced apoptosis in HCT116 colon cancer cells. Roscovitine induced apoptosis by activating mitochondrial pathway caspases and modulating the expression of Bcl-2 family members. Depletion of polyamines by treatment with difluoromethylornithine (DFMO) increased roscovitine-induced apoptosis. Transient silencing of ornithine decarboxylase, polyamine biosynthesis enzyme and special target of DFMO also increased roscovitine-induced apoptosis in HCT116 cells. Interestingly, additional putrescine treatment was found pro-apoptotic due to the presence of non-functional ornithine decarboxylase (ODC). Finally, roscovitine altered polyamine catabolic pathway and led to decrease in putrescine and spermidine levels. Therefore, the metabolic regulation of polyamines may dictate the power of roscovitine induced apoptotic responses in HCT116 colon cancer cells.

Keywords Polyamines · Apoptosis · Roscovitine · DFMO · Colon carcinoma

E. D. Arısan · A. Çoker · N. Palavan-Ünsal (✉)
Molecular Biology and Genetics Department, Istanbul Kultur University, Science and Literature Faculty, Atakoy Campus, 34156 Istanbul, Turkey
e-mail: n.palavanunsal@iku.edu.tr

Abbreviations

APAO	N^1-acetylpolyamine oxidase
CDKs	Cyclin dependent kinases
CST	Cell signaling technology
DFMO	D,L-α-difluoromethylornithine
DMSO	Dimethyl sulfoxide
H_2-DCFDA	2′-7′ Dichlorofluorescein
IEC-6	Intestine epithelial cells of rats
MMP	Mitochondrial membrane potential
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NAC	N-acetyl cystein
ODC	Ornithine decarboxylase
PAs	Polyamines
PAO	Polyamine oxidase
POD	Peroxidase
Put	Putrescine
SMO	Spermine oxidase
ROS	Reactive oxygen species
RT	Reverse transcriptase
Spd	Spermidine
Spm	Spermine
SSAT	Spermidine/spermine N1-acetyltransferase
5-FU	5-Fluorouracil

Introduction

The natural polyamines (PAs) putrescine (Put), spermidine (Spd) and spermine (Spm) are ubiquitous polycationic amine derivatives and found in all eukaryotic cells (Marton et al. 1991; Marton and Pegg 1995). Polyamines are considered essential elements for cell proliferation, differentiation and growth in normal and cancer cells (Bachrach

1976; Andersson and Heby 1977; Heby et al. 1978). Their cellular levels are referred as critical regulators in cell cycle, survival and death mechanisms (Monti et al. 1996). Polyamine metabolic regulation is characterized by several enzyme activities. Ornithine decarboxylase (ODC) is a rate limiting enzyme which induces synthesis of Put from L-arginine (Wang and Casero 2006). High accumulation of PAs in the cells are regulated by polyamine catabolic pathway players, spermidine/spermine N1-acetyltransferase (SSAT), spermine oxidase (SMO) and polyamine oxidase (PAO). These enzymes induce excretion of acetylated polyamine derivatives or provide a back-conversion pathway by oxidizing several compounds in the cells (Casero and Pegg 2009). Previous reports showed that polyamine depletion by specific inhibitor D,L-α-difluoromethylornithine (DFMO) treatment might increase the apoptotic efficiency of drugs (Choi et al. 2000; He et al. 2000; Yuan et al. 2002; Fischer et al. 2003; Hudson et al. 2003; Stanic et al. 2006; Xie et al. 2008). Therefore, polyamine metabolic pathway targeted therapies are gaining growing importance to increase the combination therapy efficiency in clinics (Chen et al. 2006; Burns et al. 2009). However, the molecular mechanism involved in drug-induced apoptosis related to polyamine biosynthetic regulation is not fully understood yet.

Roscovitine (2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopro-pylpurine) is a new generation reversible inhibitor of cyclin dependent kinases (CDKs) such as Cdc2, Cdk2, Cdk5, and Cdk7 by behaving as a competitor for ATP binding (Iseki et al. 1997; Meijer et al. 1997; Schutte et al. 1997; Wesierska-Gadek et al. 2008a). Roscovitine triggers cell cycle arrest at G_1 and G_2/M phases (Meijer et al. 1997) and leads to apoptotic induction which can be determined in every phases of cell cycle (David-Pfeuty 1999). It was also shown that following roscovitine treatment, DNA synthesis is inhibited and induces apoptosis by causing nucleolar fragmentation in various cancer cells (Aldoss et al. 2009; Hui et al. 2009; Maurer et al. 2009; Mohapatra et al. 2009; Wesierska-Gadek et al. 2009; Zhang et al. 2009; Zolnierczyk et al. 2009). Because of these cell growth-inhibiting activities, roscovitine is being considered as a potential anticancer agent and a promising candidate for combination treatment models (Ortiz-Ferron et al. 2008; Wesierska-Gadek et al. 2008b; Zhang et al. 2008; Appleyard et al. 2009). In vitro studies revealed that roscovitine is a promising therapeutic agent by inducing apoptosis in prostate cancer (Mohapatra et al. 2009), breast cancer (Appleyard et al. 2009; Maurer et al. 2009), lung cancer (Zhang et al. 2008; Aldoss et al. 2009), leukemia cells (Zolnierczyk et al. 2009). Roscovitine has also been shown as a sensitizing drug in combination with other conventional therapeutic options in the treatment of cancers (Ortiz-Ferron et al. 2008; Zhang et al. 2008).

In the present study, we have demonstrated that polyamine depletion by DFMO treatment augments the roscovitine-induced apoptosis by activating mitochondrial death pathway in HCT116 colon carcinoma cells. This is the first study that reveals the polyamine metabolism plays a critical role in roscovitine-induced apoptosis in cancer cells.

Materials and methods

Chemicals, antibodies and primers

ODC inhibitor DFMO was purchased from Tocris Biosciences (Bristol, UK). Solutions of DFMO were prepared before each experiment (dissolved in DMSO as 100 mM stock). Roscovitine (Sigma, St. Louis, MO, USA) was dissolved in DMSO to make a 10 mM stock solution and stored at −20°C. Pifithrin-α was purchased from Alexis Biosciences (Lausen, Switzerland). N-acetyl cystein (NAC) and 2′-7′ dichlorofluorescein (H_2-DCFDA, Calbiochem San Diego, CA, USA) were purchased from Calbiochem (San Diego, CA, USA). Put, Spd and Spm standards were purchased from Sigma (St. Louis, MO, USA).

Bcl-2 (1:1,000), Bcl-x_L (1:2,000), Mcl-1 (1:1,000), Bax (1:2,000), Bad (1:1,000), Puma (1:1,000), Bim (1:1,000), COXIV (1:500), β-actin (1:2,000), PARP (1:1,000), cleaved PARP (1:1,000), cleaved caspase-3 (1:1,000) and pro-caspase-3 (1:1,000), cleaved caspase-9 (1:1,000) and pro-caspase-9 (1:1,000) anti-rabbit antibodies were purchased from Cell Signaling Technology (CST, Danvers, MA, USA). Cyt c anti-mouse antibody (1:1,000) from Biovision (Palo Alto, CA, USA) and ODC, SSAT and PAO anti-rabbit antibodies (1:2,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated secondary anti-rabbit and anti-mouse antibodies (1:5,000) were from CST (Danvers, MA, USA). The appropriate primers for quantitative RT-PCR experiments were purchased from Geneglobe (Qiagen, Heidelberg, Germany).

Cell culture

HCT116 cells (CCL-247, ATCC) were maintained in McCoy medium (PAN Biotech, Aidenbach, Germany) with 2 mM L-glutamine, 10 % fetal calf serum (PAN Biotech, Aidenbach, Germany), 1 % non-essential amino acids (Biological Industries, Kibbutz Beit-Haemek, Israel), and 100 U/100 mg/ml penicillin/streptomycin (Biological Industries, Kibbutz Beit-Haemek, Israel) and grown in the presence of 5% CO_2 in humidified air at 37°C.

Cell viability assay

Cells were seeded in 96-well plates and co-treated for 24 h with various concentrations of roscovitine (0–50 μM) and ODC specific inhibitor DFMO (1–10 mM). In addition, cells were also pre-treated with DFMO for 5 min–4 h followed by roscovitine treatment. Cytotoxic effect of co- and pre-treatment efficiency for both drugs was determined by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Roche, Indianapolis, IN, USA) cell proliferation assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. Absorbance was determined at 570 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

Apoptosis determination

Cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) were determined according to manufacturer instructions by Cell Death Detection EL-ISAPLUS (Roche, Indianapolis, IN, USA). Briefly, cell lysates were placed in a streptavidin-coated microplate. A mixture of anti-histone–biotin and anti-DNA–POD was added and incubated for 2 h at 15–25°C. After removal of unbound antibodies by a washing procedure, POD was determined photometrically at 405 nm with ABTS as substrate. In order to determine the DNA fragments following drug treatment in colon carcinoma cells, total DNA content was isolated.

Cell cycle analysis

Cell cycle was analyzed after 24 h of treatment. Briefly, cells were harvested by trypsinization and washed with ice cold 1× PBS. They were fixed in ice-cold 70% ethanol, washed, resuspended in PBS and treated with RNase A (Fermentas, Glen Blunie, MD, USA). Finally, cells were stained with propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) and analyzed by flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA, USA). DNA content was quantified using CellQuest Pro software (BD Biosciences, San Jose, CA, USA).

Mitochondrial membrane potential assay

10^5 HCT116 cells were seeded in 12-well plates, allowed attaching overnight, and treated with desired concentrations of roscovitine and/or DFMO for 24 h. Cells were washed once with PBS, and then stained with Rhodamine 123 (Molecular Probes, Eugene, OR, USA) fluorescent probe. Mitochondrial membrane potential (MMP) loss was measured by Fluoroskan Ascent fluorometer (Thermo Labsystems, Beverly, MA, USA) (excitation/emission = 488 nm/525 nm).

RNA extraction and reverse transcriptase assay

Total RNA was isolated using a TRIpure reagent (Roche, Indianapolis, IN, USA) following the procedure described by the manufacturer. Total RNA was digested with RNase-free DNase (Boehringer, Mannheim, Germany) for 15 min at 37°C.

Reverse transcription (RT) was performed according to manufacturer instructions using a specific RT kit (iScript, Bio-Rad, Hercules, CA, USA). The quantity of cDNA was calculated using spectrophotometer by determination of optical density at 260 nm (OD260). Purity was calculated using the OD260/280 ratio.

Preparation of siRNA transfection

HCT116 colon carcinoma cells were placed in a six well plate 24 h prior to transfection. Cells were transfected with 20 nM ODC siRNA (Gene Globe, Qiagen, Heidelberg, Germany) using 1:6 ratio siRNA specific transfection reagent (RNAifect, Qiagen, Heidelberg, Germany) following the manufacturer's protocol. After 48 h incubation, the silencing effect was checked with qRT-PCR and western blot analysis.

Real time RT-PCR

Real-time PCR was performed in 96-well 0.2 ml thin wall PCR plates using the iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) and carried out using the SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA) as mentioned before (Arisan et al. 2009).

Immunoblot analysis

HCT116 colon carcinoma cells were treated with the appropriate concentrations of drugs. First, all samples were washed with ice-cold PBS and lysed on ice in a solution containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, Nonidet P-40 0.5 %, (v/v), 1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, protease inhibitor cocktail (Complete, Roche, Indianapolis, IN, USA). After cell lysis, cell debris was removed by centrifugation for 15 min at 13,200 *g*, and protein concentrations were determined with a Bradford protein assay. Sub-cellular mitochondrial and cytosolic protein lysates were isolated according to manufacturer instructions (Biovision Mitochondria/Cytosolic fractionantion kit, Palo Alto, CA, USA). Total protein lysates (30 μg) and mitochondria fractions (10 μg) were separated on a 12% SDS-PAGE and transferred onto PVDF

membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). The membranes were then blocked with 5% milk blocking solution in Tris buffer saline (TBS)-Tween20 (Sigma Ultra, St. Louis, MO, USA) and incubated with appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (CST, Danvers, MA, USA) in antibody buffer containing 5% (v/v) milk blocking solution. After washes with TBS-Tween 20, proteins were analyzed using an enhanced chemiluminescence detection system (ECL or ECL-Advance, Amersham Pharmacia Biotech, Little Chalfont, UK) and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech, Little Chalfont, UK).

Determination of reactive oxygen species

For assessment of reactive oxygen species (ROS) generation by different drugs in the cell cytotoxicity experiment, 2×10^5 cells were grown on 12 well plates and the H_2-DCFDA (Calbiochem San Diego, CA, USA) assay was used. Following drug treatment, HCT116 colon cancer cells were washed with 1× PBS and H_2-DCFDA (5 μg/ml) (Calbiochem, San Diego, CA, USA) in PBS was added into each well. After 30 min, PBS buffer containing excess DCF was removed from the cells and replaced with fresh PBS. Fluorescent intensity was also analyzed by Fluoroskan Ascent fluorometer (Thermo Labsystems, Beverly, MA, USA).

Statistical analysis

All samples were evaluated statistically using an Excel calculation file. Relative expression of mRNA was shown as mean ± standard deviation, and the student's *t* test was applied to understand the probability efficiency. Differences were regarded as statistically significant at values of $P < 0.05$.

Results and discussion

DFMO increases the cytotoxic effect of roscovitine in HCT116 colon cancer cells

Roscovitine is a potent apoptotic inducer in various cells such as breast, prostate, lymphoma cells and macrophages (Zhang et al. 2008; Aldoss et al. 2009; Appleyard et al. 2009; Zolnierczyk et al. 2009). The exposure of cells with various concentrations of roscovitine led to modulation of different CDKs in a dose dependent manner (Hahntow et al. 2004). In this study, HCT116 colon cancer cells were treated with roscovitine at different concentrations (0–50 μM) for 24 h. Roscovitine decreased cell viability in dose-dependent manner (Fig. 1a). According to MTT cell viability assay, 20 μM roscovitine was moderately cytotoxic and decreased cell viability of about 20%, when compared to control ($P < 0.05$* vs. untreated control). Therefore, this concentration was utilized to model roscovitine-induced apoptosis in the subsequent experiments.

In order to understand the potential role of polyamines in drug-induced apoptosis mechanism, cells were exposed to roscovitine in the presence of DFMO. Previous studies indicated that disrupted polyamine homeostasis of cells might increase the apoptotic potential of drugs in colon cancer (Choi et al. 2005) and neuroblastoma cells (Evageliou and Hogarty 2009). In addition, it was shown that DFMO increased apoptotic effect of antisense Bcl-2 RNA treatment in HL60 cells (He et al. 2000). Co-treatment with DFMO and celecoxib was found as a strong therapeutic approach in UV-induced skin carcinoma suffering mice models (Fischer et al. 2003). On the contrary, polyamine depletion might prevent tumor necrosis factor alpha-induced apoptosis in human chondrocytes (Stanic et al. 2006). Similarly, DFMO delayed camptothecin induced apoptosis by inhibiting Cyt *c* release in rat intestinal epithelial cells (IEC-6) (Yuan et al. 2002). However, both these studies were carried out on non-tumorigenic cell models. Therefore, DFMO might be a potential synergistic agent to increase drug-induced apoptosis in cancer cells and might protect the non-malignant cells from apoptotic injury.

In our experimental design, HCT116 colon cancer cells were also exposed for 24 h to 0, 1, 2.5, 5, 7.5 and 10 mM DFMO in association with roscovitine. The presence of DFMO at the concentration ranging from 2.5 to 10 mM induced a significant increase of the cytotoxic effect of roscovitine (Fig. 1a). Moreover, the time course of cell viability after treatment with 20 μM roscovitine, given alone or in combination with 5 mM DFMO, was determined (Fig. 1b).

In order to understand the time specific co-treatment efficiency, HCT116 cells were pre-treated with DFMO for different times (5 min–4 h) and then treated with 20 μM roscovitine for 24 h (Fig. 1c). DFMO pre-treatment up to 30 min significantly augmented the roscovitine induced-cytotoxicity. However, DFMO pre-treatment for 1 h followed by roscovitine did not show significant cytotoxic effect whereas pre-treatment for 2 or 4 h resulted in about 70% cell survival. Therefore, we conclude that, DFMO pre-treatment efficiency was critical in time dependent manner.

In order to confirm that DFMO acts as the specific inhibitor of ODC, we checked ODC expression profile following DFMO (5 mM) treatment in HCT116 colon cancer cells. DFMO did not alter the expression profile of ODC in HCT116 colon cancer cells (data not shown). Taken together, these results revealed that DFMO

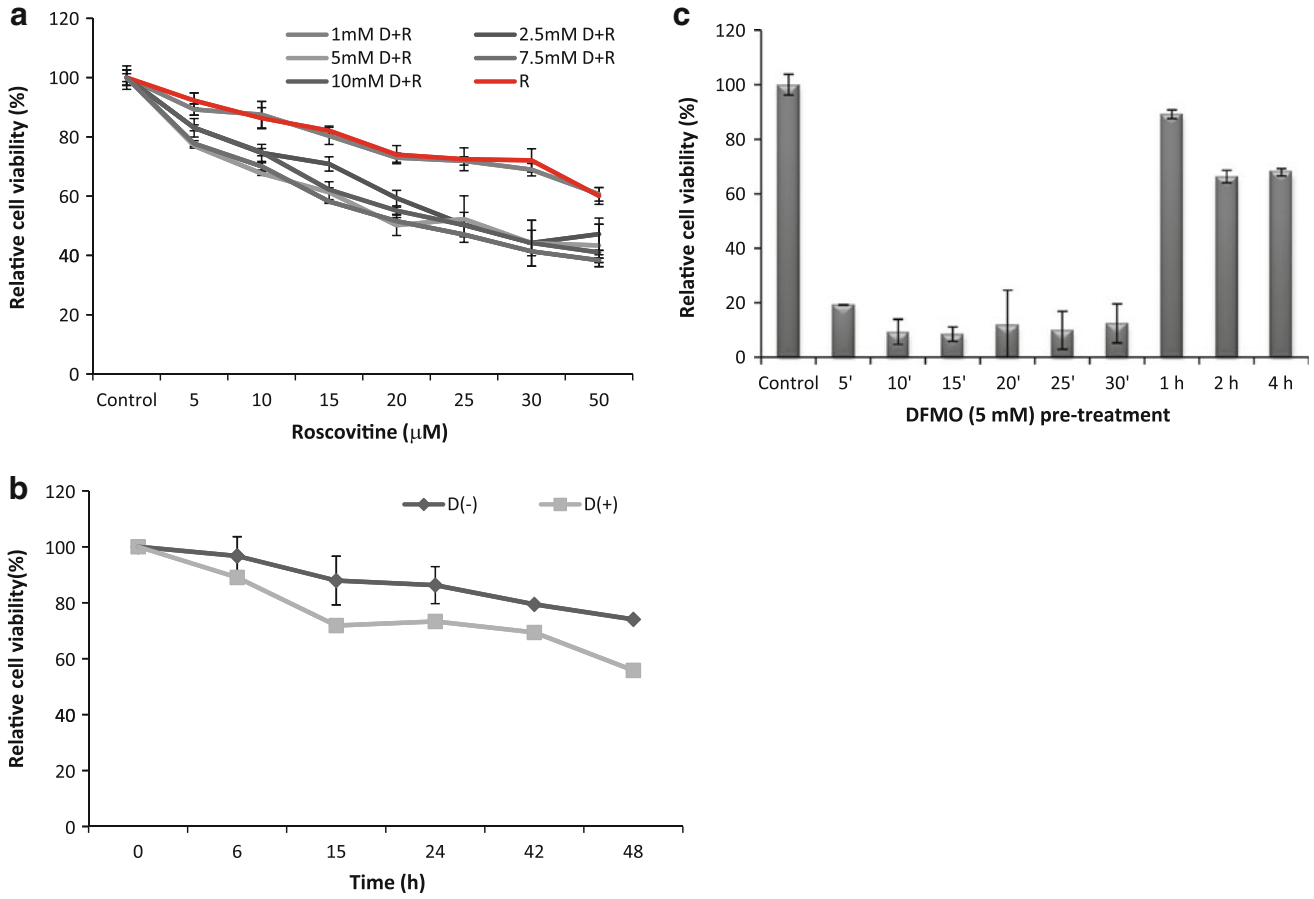

Fig. 1 Effects of the treatment with roscovitine, alone and in association with DFMO, on the cell viability of HCT116 colon carcinoma cells. **a** Dose-response curves of cell viability determined by MTT assay on cells treated with roscovitine (0–50 μM) alone (*red curve*) and in the presence of various concentrations of DFMO (1-10 mM) (*gray curves*). **b** Time course of the viability of cells treated with 20 μM roscovitine, in the absence or presence of 5 mM DFMO up to 48 h. **c** Relative HCT116 cell viability after 20 μM roscovitine treatment preceded by 5 mM DFMO exposure up to 4 h. Data represent the Mean ± SD from 3 to 6 replicated experiments. *D* DFMO, *R* roscovitine, *D* + *R* co-treatment of roscovitine with DFMO

synergistically interacted with roscovitine by enhancing its cytotoxic effect in dose and time-dependent manner.

Polyamine depletion potentiates the roscovitine-induced apoptosis in HCT116 cells

To demonstrate the efficiency of combined treatment with DFMO and roscovitine on the apoptosis induction, HCT116 cells were exposed to 5 mM DFMO or 20 μM roscovitine, alone or in combination for 24 h, and examined for several apoptotic markers. Figure 2a shows that DFMO given alone did not induce DNA fragmentation while roscovitine treatment (20 μM) caused a significant increase in DNA fragmentation (***$P < 0{,}001$), revealing apoptotic induction. Such an increase was even more evident in cells treated with the combination DFMO + roscovitine (**$P < 0.01$ vs. roscovitine alone treatment).

It is also known that roscovitine is able to induce apoptosis by altering cell cycle (Wesierska-Gadek et al. 2009). Moreover, it was shown that polyamine depletion enhances dexametazone-induced cell cycle arrest at G1 phase and apoptosis in human T lymphoblastic leukemia cells (Choi et al. 2000). In our study, cell cycle analysis revealed the presence of apoptotic cell death (subG1 group) following treatment with 20 μM roscovitine for 24 h (Fig. 2b). Although DFMO showed to induce a significant arrest in S phase in smooth muscle cells (Odenlund et al. 2009), the presence of DFMO slightly increases the roscovitine induced S phase arrest in our cell model.

A well-known effect of the apoptosis induction is the reduction of mitochondrial membrane potential (MMP). After treatment of HCT116 cells with DFMO alone, just a slight and not statistically significant ($P = 0.148$ vs. control) MMP decrease was revealed (Fig. 2c). As expected, roscovitine treatment induced a remarkable depolarization after mitochondrial membrane. Such an effect was greater in DFMO + roscovitine treated cells ($P < 0.0001$ vs. control).

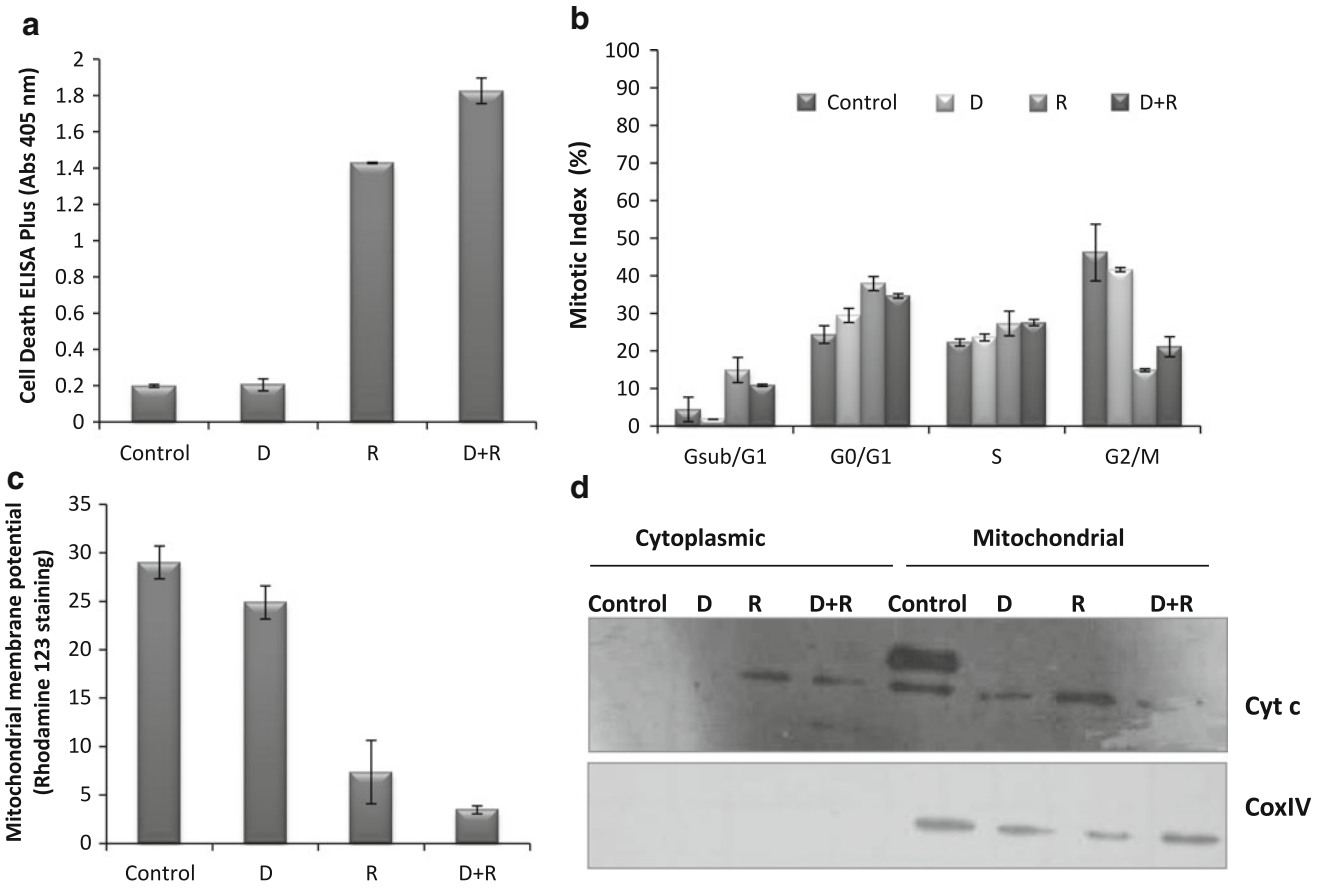

Fig. 2 Effect of DFMO on roscovitine-induced apoptosis. **a** DNA fragmentation induced by roscovitine in HCT116 colon carcinoma cells. Cells were treated with 5 mM DFMO or 20 μM roscovitine, alone or in combination, for 24 h. **b** Cell cycle analysis carried out by flow cytometry on cells treated with 5 mM DFMO or 20 μM roscovitine or the association DFMO + roscovitine. **c** Effect of the various treatments on the mitochondrial membrane potential (MMP) revealed by flow cytometric analysis on HCT116 cells stained with rhodamine 123 (5 mM for 30 min). Results were obtained from two different culture conditions with at least three replicates. **d** Cytochrome *c* release in HCT116 cells determined by western blotting. Cytoplasmic and mitochondrial proteins were extracted by mitochondrial fractionation kit and separated in 12% SDS gel, blotted in PVDF membrane and applied with Cyt *c* antibody. Cox IV antibody has been used to control mitochondrial loading

In order to confirm the apoptotic effect of roscovitine, we determined Cyt *c* release in HCT116 colon cancer cells. Previous studies indicated that roscovitine-induced apoptosis occurred through the activation of mitochondrial pathway. In addition, executioner Bcl-2 family member proteins, Bax and Bak, were found to play an important role in roscovitine-induced apoptosis (Garrofe-Ochoa et al. 2008). The synergistic effect of DFMO was found in association with activated mitochondrial pathway players of apoptosis in HL60 cells (Xie et al. 2008). According to these previous findings, we found that roscovitine in the presence or absence of DFMO, induced Cyt *c* release into cytosol from mitochondria (Fig. 2d). Furthermore, exposure of HCT116 cells to roscovitine-induced activation of caspase-9 and caspase-3. Although DFMO did not activate the proteolytic cleavage of these two caspases, it potentiated the roscovitine-induced caspase cleavage (Fig. 3a).

The roscovitine-induced apoptosis was further investigated by detection of PARP degradation. Roscovitine, in the presence or absence of DFMO, significantly induced PARP cleavage in HCT116 cells (Fig. 3b).

Functional p53 protein level was suggested as an efficient enhancer in roscovitine induced-apoptosis in cancer cells (Paprskarova et al. 2009). Roscovitine-induced apoptosis was shown to be p53-dependent in MCF-7 cells (Wesierska-Gadek et al. 2009), whereas roscovitine might induce apoptosis in B-CLL cells regardless of the functional status of the p53 pathway, and it might be considered as a therapeutic agent to improve the outcome of resistant B-CLL tumors (Alvi et al. 2005). Therefore, the possible relationship between roscovitine and p53 regulation is not fully understood. In addition, synergistic effect of DFMO with 5-Fluorouracil (5-FU), a conventional colon carcinoma chemotherapeutic agent, was shown in p53 (wt) and

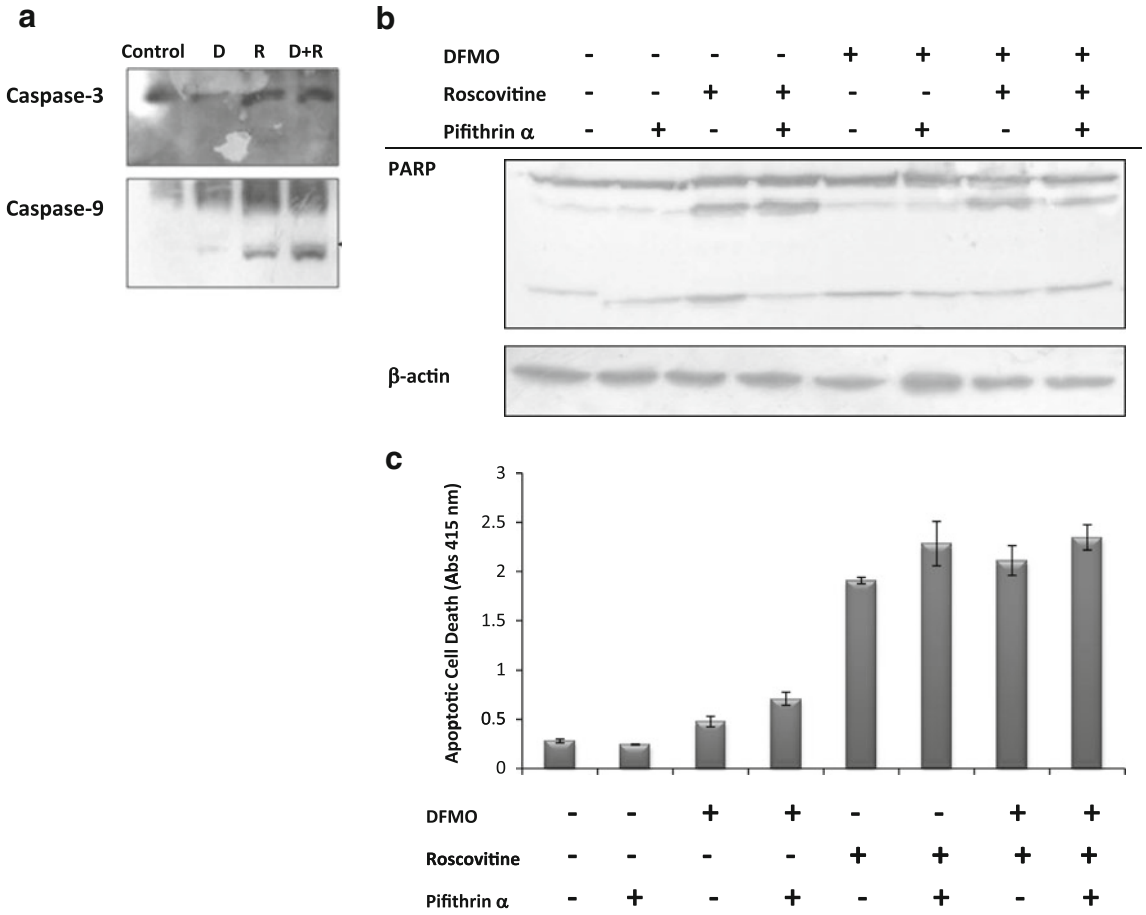

Fig. 3 Roscovitine-induced apoptosis results to be caspases-dependent and p53- independent. **a** Caspase-3 and caspase-9 expression profiles determined by immunoblotting in cells treated with roscovitine, DFMO and their combination for 24 h. **b** 12 % SDS-PAGE gel was utilized to perform PARP immunoblotting on cells treated with roscovitine, DFMO, 30 μM pifithrin-α (a p53 specific inhibitor) or their combination for 24 h. Co-treatment with the p53 specific inhibitor did not prevent PARP cleavage. *β*-actin was used as loading control. **c** DNA fragmentation in HCT116 cells following administration of pifithrin-α in association with DFMO and/or roscovitine. The reported values represent the Mean ± SD of two individual experiments with two replicates

p53 (null) HCT116 cells (Choi et al. 2005). In order to understand the potential role of p53 in roscovitine induced apoptosis, HCT116 cells (p53 wt) were pretreated with pifithrin-α (Bragado et al. 2007), a specific inhibitor of p53. Inhibition of p53 did not alter roscovitine-induced PARP cleavage (Fig. 3b). This result was also confirmed by detection of DNA fragments in HCT116 cells following administration of pifithrin-α in association with DFMO and/or roscovitine (Fig. 3c). Therefore, we conclude that roscovitine-induced apoptosis did not depend on active p53. However, further experiments will be required by using p53 mutant cells to put forward potential role of p53 in roscovitine-induced apoptosis.

Roscovitine modulates Bcl-2 family members in HCT116 colon carcinoma cells

Since Bcl-2 is known to protect the cells against various apoptotic stimuli including chemotherapeutic drugs (Kutuk et al. 2009), we determined the modulation of Bcl-2 family members following roscovitine treatment in the presence or absence of DFMO for 24 h. Although roscovitine significantly down-regulated anti-apoptotic Bcl-2 and Mcl-1 expression in HCT116 cells, no alteration in Bcl-x_L expression profile was revealed (Fig. 4). According to these findings, roscovitine was shown to trigger apoptosis by inhibiting de novo transcription of the short-lived Mcl-1 in neutrophils (Leitch et al. 2010) and B-CLL cells (Hallaert et al. 2007). Interestingly, we did not observe any significant synergistic effect of DFMO in roscovitine-altered Bcl-2 family expression profile.

The pro-apoptotic Bcl-2 family members; Bax, Puma-α and Bim were found up-regulated following roscovitine treatment in the presence or absence of DFMO. However, roscovitine did not alter Bad expression in HCT116 cells.

It was established that roscovitine-induced accumulation of p53 regulates the Puma transcription in the cells but Puma or Noxa, which are p53 transcriptional targets, were

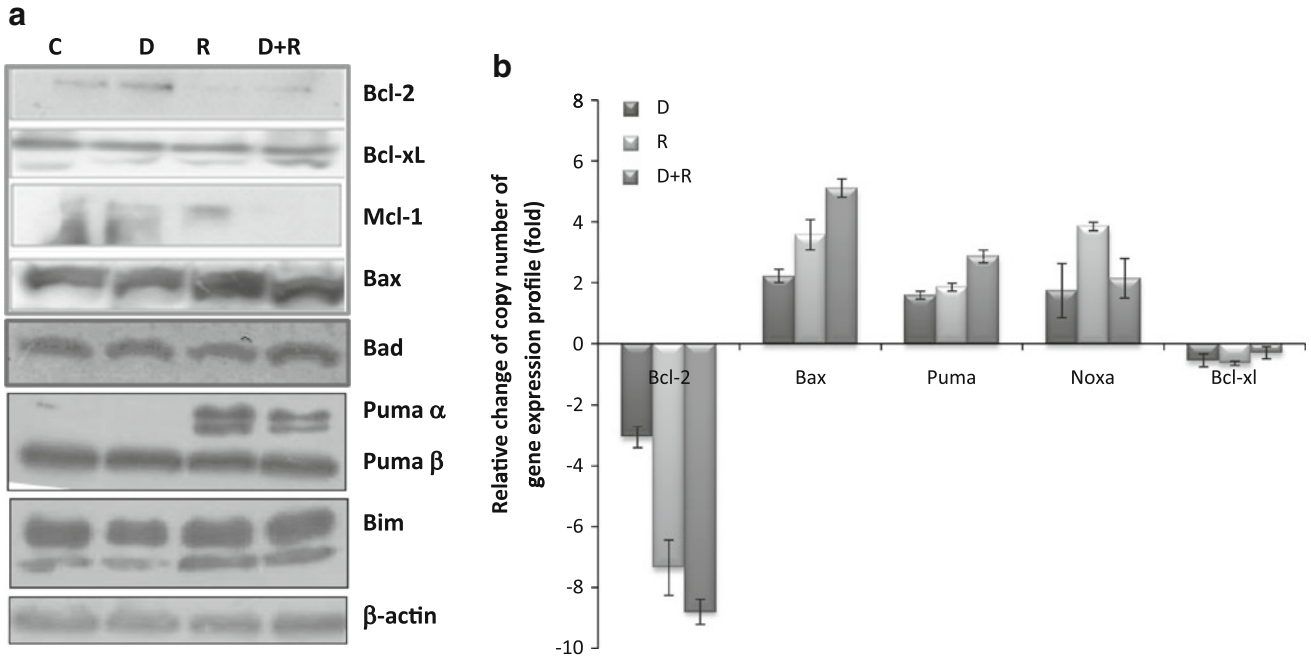

Fig. 4 Modulation of the Bcl-2 family members induced by roscovitine. **a** Expression of Bcl-2 family members detected by immunoblotting following exposure of cells to roscovitine, DFMO and their combination for 24 h. Total protein content was isolated and then quantified according to Bradford method. β-actin was used as loading control. **b** Total mRNA content was evaluated for Bcl-2, Bax, Puma, Noxa and Bcl-x_L. The values represent the Mean ± SD of two individual experiments with two replicates

shown to be regulated independently on p53 in various cells models (Chen et al. 2007; Cheok et al. 2007). HCT116 colon carcinoma cells (p53 wt) showed up-regulation of Puma-α after treatment with DFMO + roscovitine. Although Noxa is a transcriptional target of p53, DFMO did not increase the roscovitine-induced Noxa up-regulation (Fig. 4). Therefore, we again conclude that functional regulation of p53 in apoptosis should be checked in further studies by utilizing p53 mutant colon carcinoma cells.

Functional role of intracellular polyamine content in roscovitine-induced apoptosis

In order to evaluate the potential role of polyamines in roscovitine-induced apoptosis, their content has been determined in cells treated with roscovitine, alone and in association with DFMO.

As shown in Fig. 5a. DFMO remarkably decreased Put and Spd levels in HCT116 colon cancer cells, whereas no significant effect was observed in Spm content. Roscovitine given alone slightly decreased the levels of three PAs. When the cells were exposed to DFMO +roscovitine, the Put level was further decreased ($^*P < 0.05$), indicating an additive effect of the two compounds. Therefore, Spd and Spm levels did not exhibit the same trend after the co-treatment of both drugs.

To evaluate the possible relationship between decreased Put content and roscovitine-induced apoptosis, HCT116 colon cancer cells were treated with DFMO, roscovitine or DFMO + roscovitine in the presence or absence of exogenous 10 μM Put (Fig. 5b) for 24 h. In disagreement with previous reports (Hudson et al. 2003), the exogenous Put treatment increased the synergistic effect of DFMO on roscovitine-induced apoptosis in our experimental model.

In addition, in order to understand the functional role of ODC in the programmed cell death, the enzyme was transiently silenced prior to drug treatment. We observed that the specific down-regulation of ODC increased roscovitine-induced apoptosis in HCT116 cells by fourfold, compared to parental cells (Fig. 5c—$^{**}P < 0.001$). Although additional Put treatment enhanced the synergistic effect of ODC inhibitor on roscovitine-induced apoptosis, Put did not exert the same effect in ODC silenced HCT116 colon cancer cells (Fig. 5c). Therefore, we might deduce that specific inhibition of ODC by DFMO treatment caused aberrant accumulation of Put in the cells. Cells might increase the transport efficiency of Put following DFMO treatment to overcome Put deficiency. However, down regulation of ODC completely block the Put biosynthesis and also block the Put transportation from extracellular environment. Thus, additional searches will be required to clarify the functional role of natural polyamine inhibitor, antizyme and its regulation (Wang et al. 2007) following roscovitine treatment.

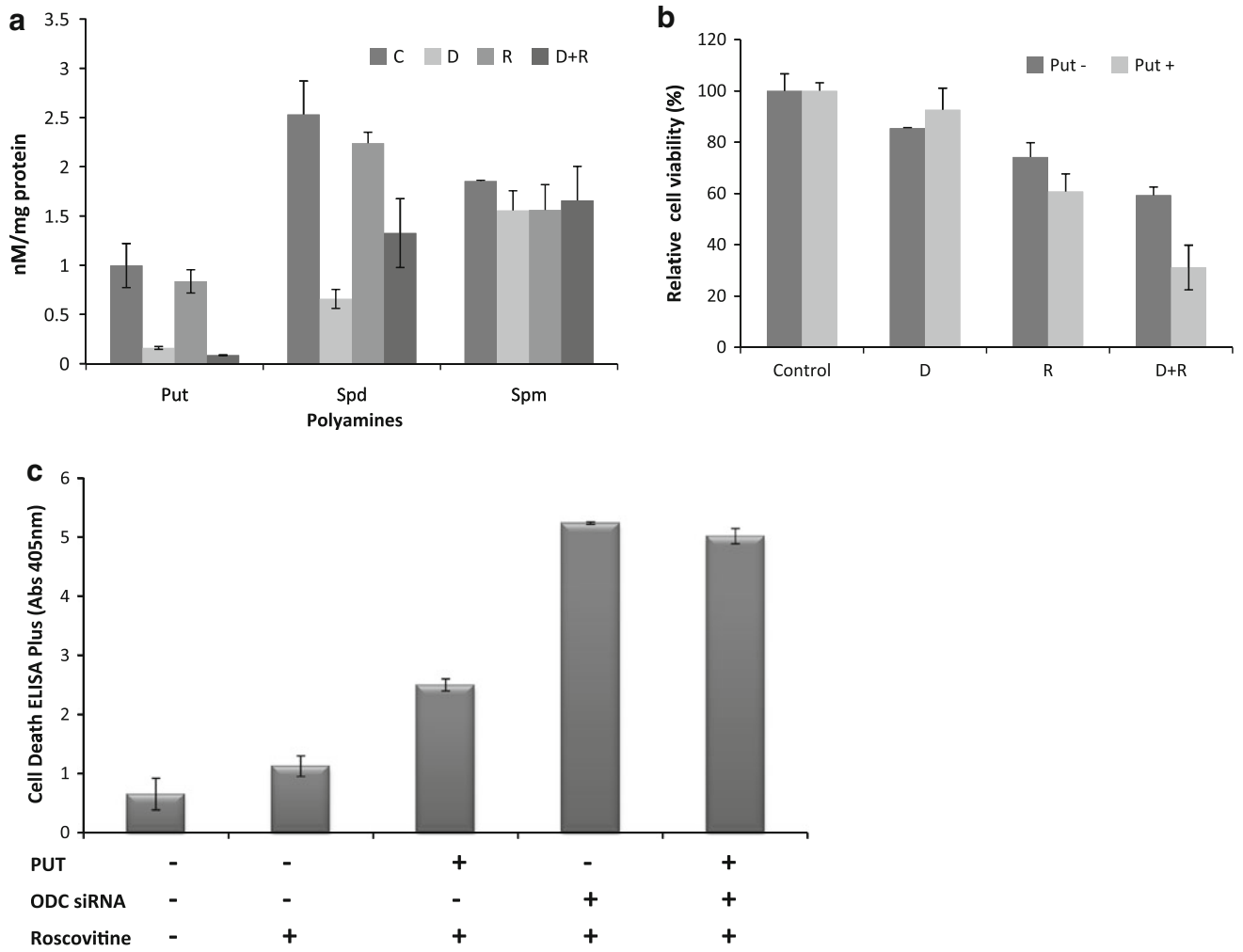

Fig. 5 Role of intracellular polyamine content in roscovitine-induced apoptosis. **a** Polyamine depletion following roscovitine treatment, alone and in association with DFMO, for 24 h determined by HPLC analysis in HCT116 colon cancer cells. *Put* Putrescine, *Spd* Spermidine, *Spm* Spermine. **b** Roscovitine-induced apoptosis was increased by the treatment with additional 10 μM putrescine. **c** Apoptotic cell death determined in ODC transiently silenced HCT116 cells by 48 h, and in cells treated for 24 h with 10 μM putrescine or 20 μM roscovitine

Roscovitine alters polyamine catabolic pathway and increases reactive oxygen species generation

In this study, we have found for the first time that roscovitine, an inhibitor of CDKs, induces in HCT116 cells a significant increase of the expression profile of the catabolic enzymes SSAT and PAO (Fig. 6a). The specific inhibition of ODC by DFMO treatment increased roscovitine-induced PAO up-regulation with release of toxic products such as H_2O_2 and reactive aldehydes (Agostinelli et al. 2006, 2010; Wang and Casero 2000). Thus, it has been evaluated if the drug-induced up-regulation of PAO and SSAT can influence the production of ROS. Roscovitine, in the presence or absence of DFMO, induced generation of ROS and this effect was partially inhibited by the addition of NAC, a scavanger for H_2O_2 (Fig. 6b). In agreement with previous findings, we figure out that drug-induced polyamine catabolic pathway might be critical for mitochondrial apoptosis by elevating ROS levels in the cells (Agostinelli et al. 2007). Since spermine oxidase was shown as the major source of H_2O_2 in the cells, it will be required to investigate the polyamine catabolic enzyme activities, SMO and N^1-acetylpolyamine oxidase (APAO), to reveal the potential role of PA metabolism in CDK inhibitors-induced apoptosis.

Conclusion

In the present paper, we have confirmed that roscovitine is a potential apoptotic drug and have demonstrated that polyamine depletion might be critical in roscovitine-

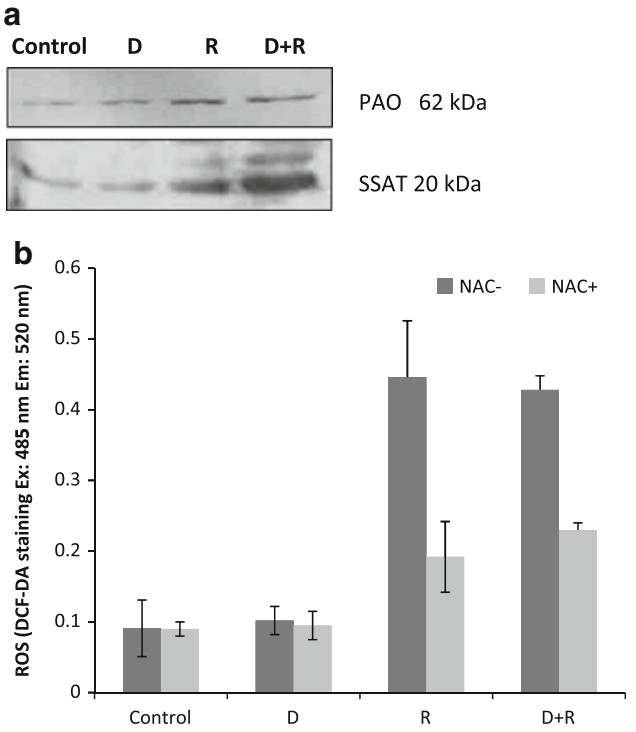

Fig. 6 Expression of polyamine catabolic enzymes and ROS production in treated HCT116 cells. **a** Drug-modulation of the polyamine catabolic enzymes (PAO, SSAT) determined by immunoblotting in HCT116 cells. *SSAT* Spermidine/spermine-N-acetyl transferase, *PAO* Acetylpolyamine oxidase. **b** ROS generation revealed by DCF-DA staining in HCT116 cells after treatment with DFMO, roscovitine or DFMO + roscovitine, in the absence or in the presence of 50 μM NAC

induced apoptosis. Roscovitine activates mitochondrial pathway by modulating anti-apoptotic molecules Bcl-2 and Mcl-1 and the pro-apoptotic Puma. Exposure of cells to roscovitine decreases intracellular polyamine levels and induces increase of catabolic pathway members, such as SSAT and PAO. Therefore, increased ROS generation related to up-regulated polyamine catabolism might be critical in roscovitine-induced apoptosis. Previous and our findings provide a biological rational for using roscovitine, a new generation CDK inhibitor, in the treatment of colon carcinoma. According to these findings, modulation of intracellular polyamine levels might augment the therapeutic efficiency of roscovitine.

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