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Metabolic effects of the HIV protease inhibitor – saquinavir in differentiating human preadipocytes

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Abstract:
Background: The iatrogenic, HIV-related lipodystrophy is associated with development of the significant metabolic and cardiovascular complications. The underlying mechanisms of antiretroviral (ARV) drugs are not completely explored.
Methods: The aim of the study was to characterize effects of the protease inhibitor (PI) – saquinavir (SQV) on metabolic functions, and gene expression during differentiation in cells (Chub-S7) culture.
Results: SQV in concentrations observed during antiretroviral therapy (ART) significantly decreased mitochondrial membrane potential (MMP), oxygen consumption and ATP generation. The effects were greater in already differentiated cells. This was accompanied by characteristic changes in the expression of the genes involved in endoplasmic reticulum (ER) stress, and differentiation (lipid droplet formation) process such as: *WNT10a, C/EBPα, AFT4, CIDEC, ADIPOQ, LPIN1*.
Conclusions: The results indicate that SQV affects not only metabolic (mitochondrial) activity of adipocytes, but affects the expression of genes related to differentiation and to a lesser extent to cell apoptosis.

Key words:
HIV-associated lipodystrophy, protease inhibitors, adipocyte, oxidative stress, mitochondrial toxicity, apoptosis, gene expression

Abbreviations: *ADIPOQ* – adiponectin C1Q, *AFT4* – Activating Transcription Factor 4, ART – antiretroviral therapy, ARV – antiretroviral, *C/EPBα* – CCAAT/enhancer binding protein α, ER-stress – endoplasmic reticulum stress, FSC – fetal calf serum, HIV – human immunodeficiency virus, LD – lipid droplet, LDH – lactate dehydrogenase, *LPIN1* – lipin 1, MMP – mitochondrial membrane potential, NRTIs – nucleoside reverse transcriptase inhibitors, PCR – polymerase chain reaction, PIs – protease inhibitors, SQV – saquinavir, VLDL – very low density lipoprotein, *WNT10a* – Wingless-type MMTV integration site family member 10A

Introduction

Treatment of HIV infection with antiretroviral (ARV) drugs, effectively suppresses viral replication and leads to the significant improvement in morbidity and mortality [49]. However, ARV therapy (ART) is associated with the risk of significant side effects, including metabolic disturbances such as lipodystrophy, insulin resistance, dyslipidemia, which can occur, in ac-

cordance to some authors, even in more than 50% of patients, and result in significant cardiovascular complications [5, 49].

Lipodystrophy, congenital or iatrogenic, as this related to ART, is the second most frequent disease of adipose tissue after obesity [20]. It also causes significant psychological problems connected with decrease of self-esteem, and social stigmatization, which may result in the non-adherence to the therapy, and finally lead to the development of multiresistance and therapeutic failure [21, 49]. Lipodystrophy increases also the risk of insulin resistance and dyslipidemia-induced cardiovascular diseases, which are now responsible for more than 10% of deaths among HIV infected patients [3, 40, 49].

In spite of numerous studies, the molecular basis of ART induced lipodystrophy are not completely understood, and recent reports point to certain aspects of the pathophysiology of adipose tissue, which up to now were not taken into consideration [21, 40]. It is suggested that development of lipodystrophy and metabolic disturbances can be related to the not yet elucidated interaction between HIV and different classes of ARV drugs, with special consideration to nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs) [21, 54]. These drugs may affect the adipocyte differentiation, mitochondrial function, ability of cells to respond properly to oxidative stress, apoptosis, or secretion of adipokines [21, 54, 60].

The aim of the study was to characterize the influence of saquinavir (SQV) (Invirase), a PI – on metabolic function and changes of differentiation-lipid accumulation-related gene expression using of human pre- and adipocytes *in vitro* [13, 14].

Methods

Cell type and culture conditions

The Chub-S7 cells [13, 14] (Nestle Research Center, Lausanne, Switzerland) were cultured in a mixture (1 : 1 (v/v) of DMEM (Sigma-Aldrich, Steinheim, Germany) and F12 (Sigma-Aldrich, Steinheim, Germany) culture media.

Differentiation procedure

For differentiation cells were plated (at a density of 20–30 × 10^3 cells/cm^2) in the above mentioned media, supplemented with 10% fetal calf serum (FCS) (Gibco, Grand Island, NY, USA) [13]. At confluence cells were incubated in a serum-free DMEM/F12 medium, supplemented with 15 mM $NaHCO_3$, 17 μM D-panthotenic acid, 15 mM Hepes and 33 μM biotin, 10 μg/ml transferrin, 1 nM triiodothyronine, 850 nM insulin, 500 μg/ml fetuin added freshly (according to protocol supplied by Nestle Research Center for Chub-S7 cultivation). This medium was referred as the "basal medium".

Preadipocyte differentiation was achieved by adding 1 μM dexamethasone and 1 μM rosiglitazone [13, 14] to the basal medium for 21 days (according to protocol supplied by Nestle Research Center).

The saquinavir (SQV, RO-31-8959/000 MRS), pure substance, was obtained from Roche Diagnostics GmbH (Mannheim, Germany). The drug stock solution (initially dissolved in ethanol and then made up to 1 mM in PBS) was stored at –20°C and diluted with the culture medium to the required concentration. The range of the used SQV concentrations (1–30 μM) was chosen on the basis of the cytotoxicity (Fig. 1) and available data describing SQV concentration detected in plasma of HIV infected patients during therapy [44]. The medium with freshly diluted SQV was changed every day.

For measurement of the effects of the drug on differentiated adipocytes, SQV was added in the above described concentrations on day 15 of differentiation and the cells then were incubated for the next 15 days. Differentiated cells incubated without SQV for next 15 days were described as Ctrl(+). Cells without differentiation factors and without SQV were incubated in the same way only with 30 μl vehicle (1 : 5 ethanol/PBS) diluted in 1 ml medium and served as negative control (Ctrl(–)).

The cytotoxicity assay

Potential cytotoxic effect of SQV on cells before and after differentiation, was determined by the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Germany) following the manufacturer instruction. The assay measures the lactate dehydrogenase (LDH) activity in the cell culture. Data were calculated as percentage of LDH activity, released into the medium

after the exposure to SQV, in comparison to the LDH activity in the negative control. The different concentrations of SQV were considered to be cytotoxic, when the amount of LDH was increased by 20% when compared to the control.

Cell differentiation assay – The Oil Red-O staining

The cellular lipid content was assessed by lipid staining with Oil Red-O [37]. For the staining 0.5 g Oil-Red-O (NAD10871, Sigma-Aldrich, Steinheim, Germany) was dissolved in 100 ml of isopropanol (POCh, Gliwice, Poland).

According to the manufacturers instruction: cells were washed twice with PBS. Cells were fixed 1 h at room temperature in 3.7% paraformaldehyde (POCH, Gliwice, Poland) in PBS without Mg and Ca ions (Biomed, Lublin, Poland). Then, paraformaldehyde solution was removed, and cells were washed shortly with doubly distilled H_2O, and stained with Oil Red-O solution for 1 h at room temperature [36]. After this time the Oil Red-O containing medium was removed. The total amount of Oil Red-O was determined by elution with 200 µl of 100% isopropanol and the spectrophotometric absorbance of the resulting solution was quantified at 500 nm using a microplate reader Multiscan (ThermoLab Systems). The total amount of Oil Red-O in each sample was then recalculated per 1 mg of protein (estimated by Lowry method) [37].

Analysis of mitochondrial function

Measurement of the mitochondrial oxygen consumption

Mitochondrial respiration assay was performed using the high-resolution respirometry system Oxygraph-2k (Oroboros Instruments, Austria). Mitochondrial oxygen consumption was monitored at 37°C in a thermostatically controlled chamber. The routine (endogenous) respiration was measured on 1×10^6 cells in 2 ml of DMEM/F12 medium. Data were digitally recorded using Dat Lab4 software where oxygen flux was calculated as the negative time derivative of oxygen concentration. Oxygen sensors were calibrated routinely at air saturation and in oxygen depleted media [23].

Mitochondrial membrane potential (MMP)

Changes of the MMP were monitored by fluorescent staining of the living cells with JC-1 dye [11].

The cells were incubated with 2 mM JC-1 solution (MitoProbe Assay Kit, Invitrogen Life Technologies, Carlsbad CA, USA) in the dark for 45 min at 37°C. Then, the cells were washed, diluted in PBS and analyzed by flow-cytometry. Autofluorescence and JC-1-induced fluorescence changes were recorded with FACSCanto flow cytometer (BD Biosciences Discovery Labware, Bedford, MA, USA) using 488 nm excitation with 530/30 nm (FL1, green) and 585/42 nm (FL2, orange) band pass emission filters. Fluorescence of 1×10^4 cells was collected during a single instrument run. The respective gates were defined using the distinctive forward-scatter and side-scatter properties of each individual cell population. The data were analyzed using the FacsDIVA software (BD Biosciences Discovery Labware, Bedford, MA, USA).

MMP was quantified by a ratio of red to green fluorescence intensities generated by JC-1 staining. This ratio is dependent only on the MMP and not on other factors such as mitochondrial size, shape, and density [12, 50]. Preincubation of cells with the protonophoric uncoupler of oxidative phosphorylation: carbonylcyanide m-chlorophenylhydrazone (CCCP) at concentration 1 µM, for 2 min was used as positive control for the JC-1 sensitivity characterizing changes of the mitochondrial membrane potential [52].

Measurement of changes of the cellular ATP content

The intracellular ATP content was measured by using the luminescence assay ATPLite™ (Perkin Elmer, Waltham, MA, USA) system based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. Briefly, cells growing on a 6-well plate were washed twice with PBS, treated with mammalian cell lysis solution, and shaken for 5 min in an orbital shaker at 700 rpm to lyse cells and stabilize the ATP content. Then, 150 µl of cell lysate was transferred to a 96-well plate and 50 µl of substrate solution, containing luciferase/luciferin, added to each well. The plate was dark adapted for 10 min and the luminescence was measured using Tecan Genios microplate reader. Standardization was performed using known quantities of standard ATP provided with the kit. Data were calculated using Magel-

lan software. Protein content was measured in the samples of cell lysates by Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma, Poznań, Poland). The ATP content was adjusted for protein content (measured by Lowry method) in sample and expressed as nmol ATP/mg of cell protein.

Analysis of the relative gene expression for the selected genes

In order to confirm influence of SQV on expression of genes important for Chub-S7 cell differentiation, the quantitative expression analysis of *WNT10a* (Wingless-type MMTV integration site family, member 10A), *C/EBPα* (CCAAT/Enhancer Binding Protein α), *ATF4* (Activating Transcription Factor 4 (tax-responsive enhancer element B67)), *CIDEC* (Cell death-inducing DFFA-like effector c), A*DIPOQ* (adiponectin, C1Q) and collagen domain containing *LPIN1* (lipin 1) was performed by real-time PCR using *18S rRNA* as the reference gene.

Following incubation, total RNA was isolated from cells using TRIzol® Plus RNA Purification System (Life Technologies, Carlsbad, CA, USA). The quality of RNA was confirmed by analysis on the NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA).

One microgram of total RNA was reverse transcribed using a reverse transcription kit (High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA)) with random primers. Subsequently, cDNA was subjected to real-time PCR. Quantitave real-time polymerase chain reaction (qPCR) was performed with the QuantiTect SYBR Green PCR kit, using primers listed in Table 1. Amplification was performed using the continuous fluorescence detection system 7900 HT Fast Real Time PCR system (Applied Biosystems, Carlsbad, CA, USA). Data were obtained in a form of sigmoid amplification plots in which fluorescence was plotted against the number of cycles. The threshold cycle (C_T) served as a measurement of the starting template amount in each sample. Calculations were performed using the Calculation Matrix for PCR Efficiency software. Expression ratio was calculated as normalized C_T difference between the control probe and the sample with adjustment for amplification efficiency.

Assay of cell apoptosis

Cell apoptosis assay was performed using FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) with FITC annexin V and propidium iodide (PI) for flow cytometry.

Briefly, cells were harvested by using a solution of 0.05% trypsin with 0.02% EDTA incubated for 1 min and washed in cold phosphate-buffered saline. Then, 5 µl of FITC annexin V and 1 µl of the 100 µg/ml PI were added to each 100 µl of cell suspension 1×10^6/ml in PBS. Cells were incubated for 15 min, and then suspension was diluted with 400 µl of annexin V-binding buffer.

After staining a cell population with FITC annexin V and PI in the provided binding buffer, populations were immediately analyzed by flow cytometry using FACSCanto flow cytometer (Becton-Dickinson, Sparks, MD, USA) with the 488 nm line of an argon-ion laser for excitation and fluorescence emission at FL1 and FL3.

The cell population was separated into three groups: live cells showing no fluorescence, apoptotic cells showing green fluorescence and dead cells showing both red and green fluorescence.

Tab. 1. Sequences of primers used in real-time PCR

Gene	Forward	Reverse
18S rRNA	5'-CGGCTACCACATCCAAGGAA-3'	5'-GCTGGAATTACCGCGGCT-3'
C/EBPa	5'-AAGAAGTCGGTGGACAAGAACAG-3'	5'-TGCGCACCGCGATGT-3'
ATF4	5'-CTGTGGATGGGTTGGTCAGT-3'	5'-TCCAAGTCGAACTCCTTCAAA-3'
ADIPOQ	5'-GAGATGGCACCCCTGGTGA-3'	5'-CCCTTAGGACCAATAAGACCTGG-3'
CIDEC	5'-TCATGGCTTACAGTCTTGAGGA-3'	5'-GGGCTTGGAAGTACTCTTCTGTC-3'
LPIN1	5'- CGAGATGAGCTCGGATGAG-3'	5'- TGGAATGGAGGTATATCATTAGGAA-3'
WNT10A	5'-GGGTGCTCCTGTTCTTCCTAC-3'	5'-GAGGCGGAGGTCCAGAAT-3'

Statistical analysis

All data were expressed as the mean + SD from at least three independent experiments measured in triplicate. Data from analysis of MMP, ATP and oxygen consumption were expressed as percentage of the respective (non-differentiated or differentiated) non treated control cells. Statistical significances for comparisons between treated samples and corresponding untreated cell samples were determined using the unpaired *t*-test. All analyses were performed using Statistica 10.0 PL software.

Calculation of fold of change and statistical significances were determined using a Pair Wise Fixed Reallocation Randomisation Test using the Calculation Matrix for PCR Efficiency software. Data were presented as fold of change *vs.* respective control cells. As a reference gene, expression of *18SrRNA* was used.

Results

Cytotoxic effect of SQV

In the used experimental conditions SQV exerted the cytotoxic effect at the highest concentration of 100 μM (Fig. 1).

Influence of SQV on cell differentiation lipid accumulation (The Oil Red-O staining)

The 15-day incubation of cells in the differentiation medium resulted in the significant increase in the cellular lipid content as measured by the lipid Red-Oil staining (Fig. 2). Incubation of differentiated adipocytes with SQV, especially with the higher concentrations (> 10 μM) significantly reduced cellular lipids (Fig. 2).

In the case of undifferentiated preadipocytes no effect of SQV on the cell lipid content was seen (Fig. 2).

Influence of SQV on mitochondrial function in Chub-S7 cells

Oxygen consumption in Chub-S7 incubated with SQV

The routine rate of respiration was determined in intact cells by high resolution respirometry, using the energy substrates present in the medium. The non-differentiated and differentiated control Chub-S7 cells presented similar rate of routine respiration (33.65 *vs.* 33.96 pmol/s × ml, respectively).

The polarographic measurements showed the inhibitory effect of SQV (20 or 30 μM) on oxygen consumption in both pre- as well as adipocytes (Fig. 3). Stronger effect was noticed in differentiated adipocytes, which suggested that the mature adipocytes were more susceptible to SQV activity in terms of mitochondrial respiration disturbances.

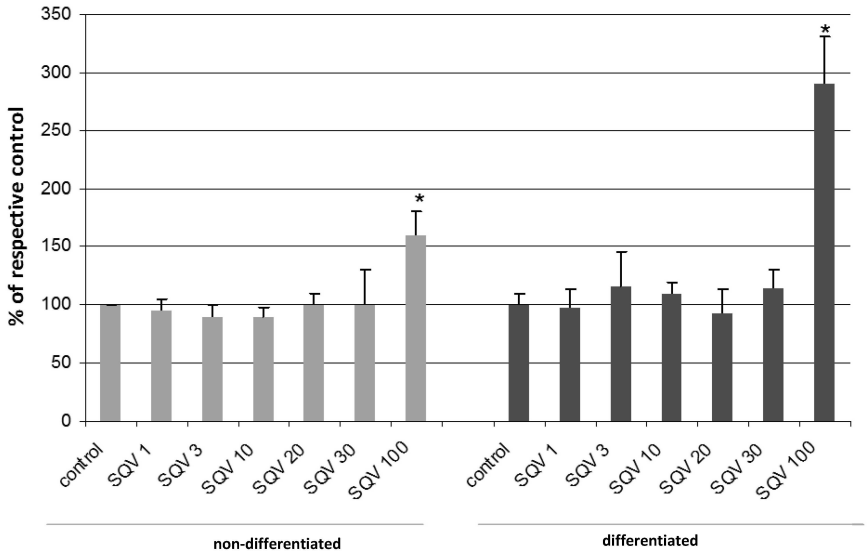

Fig. 1. The cytotoxic effect of different concentrations (1–100 μM) of saquinavir (SQV) in non-differentiated and differentiated Chub-S7 cells. Data are presented as percentage of negative control (cells incubated only with 30 μl vehicle (1 : 5 ethanol/PBS, v/v) diluted in 1 ml medium). Significance: * $p < 0.05$ *vs.* respective control performed in three independent experiments in triplicate

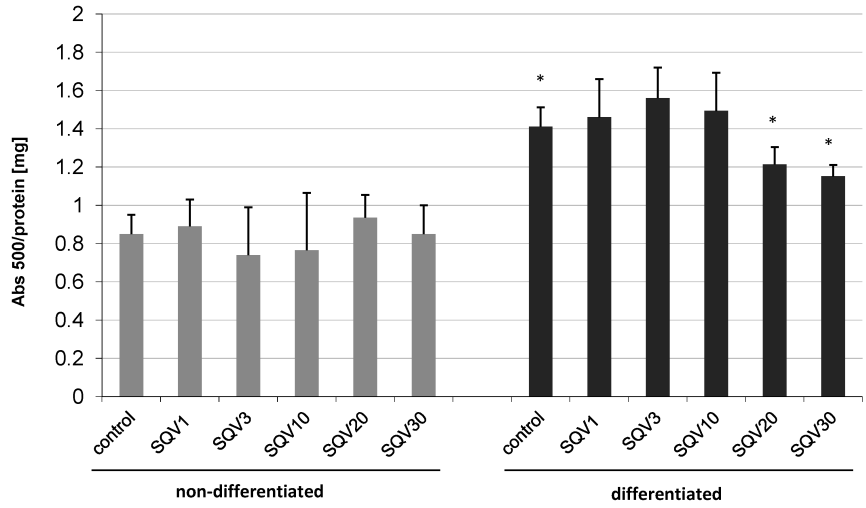

Fig. 2. Lipid accumulation (Oil-Red-O staining) in non-differentiated and differentiated Chub-S7 (after 15 days incubation) cells in the presence of saquinavir (SQV, 1–30 μM). Data are presented as the mean absorbance per mg of protein. Significance: * $p < 0.05$ *vs.* respective control, * $p < 0.05$ differentiated control cells *vs.* non-differentiated control cells performed in three independent experiments in triplicate

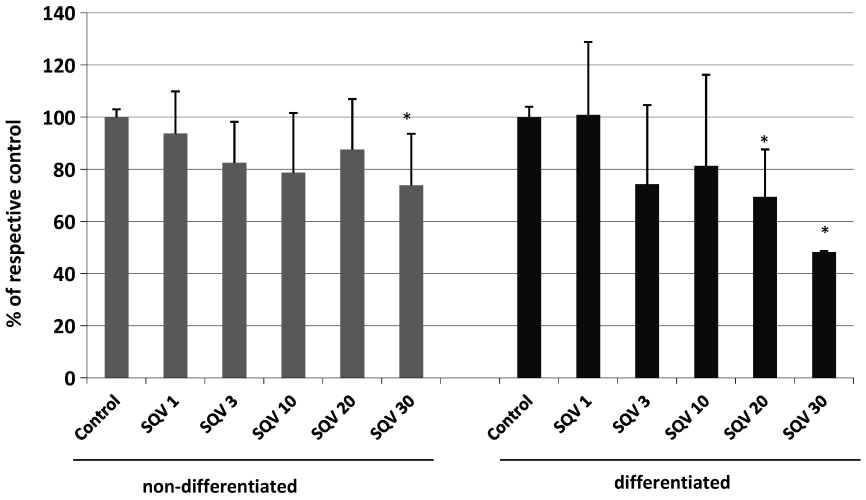

Fig. 3. Oxygen consumption in non-differentiated and differentiated Chub-S7 cells after 15 days incubation with different concentration (1–30 μM) of saquinavir (SQV). Data are presented as percentage of respective control. * $p < 0.05$ *vs.* respective control performed in three independent experiments in triplicate

Changes in MMP

The differentiated non-treated control Chub-S7 cells presented insignificantly lower inner MMP value analyzed by JC-1 staining as compared to corresponding non-differentiated control cells (100.7 ± 10.0 *vs.* 84.2 ± 14.0 respectively; n = 5, p = 0.09).

The study demonstrated the lower of MMP values potential in differentiated adipocytes incubated with SQV (20 and 30 μM) for 15 days (Fig. 4). The measured index of red to green signal (JC-1 aggregate to monomer ratio), was reduced by 20–30% in these cells in comparison to the respective non treated SQV control cells.

The opposite effect of SQV on MMP was found in non-differentiated preadipocytes. The inner mitochondrial membrane potential tended to increase by higher concentration of SQV. The observed effect on MMP indicated an increase of the proton motive force, which may result in free radical generation as well as cell death [15, 38].

Both too low or too high MMP may cause disturbances due to impaired oxidative phosphorylation process or the reactive oxygen species generation by the metabolically overloaded mitochondria [27, 31].

Cellular ATP content in Chub-S7 incubated with SQV

To verify the capability of the cell to produce ATP from the oxidative substrates, intracellular ATP content was measured. The level of ATP in non-differentiated and differentiated control preadipocytes corresponded to 104.2 *vs.* 81.0 nmol/mg, respectively

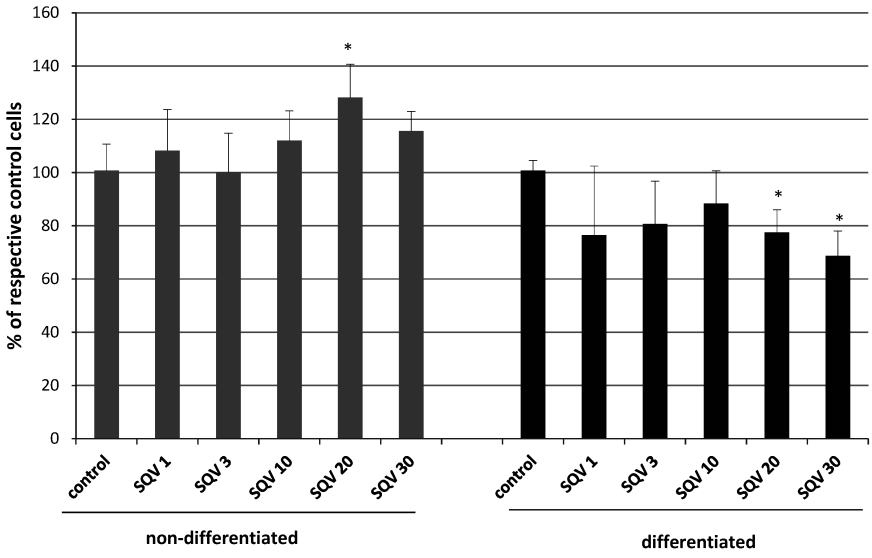

Fig. 4. Inner mitochondrial membrane potential (MMP) in non-differentiated and differentiated Chub-S7 cells after 15 days incubation with different concentration (1–30 μM) of saquinavir (SQV). Data are presented as percentage of respective control. * $p < 0.05$ *vs.* respective control performed in five independent experiments in triplicate (n = 5)

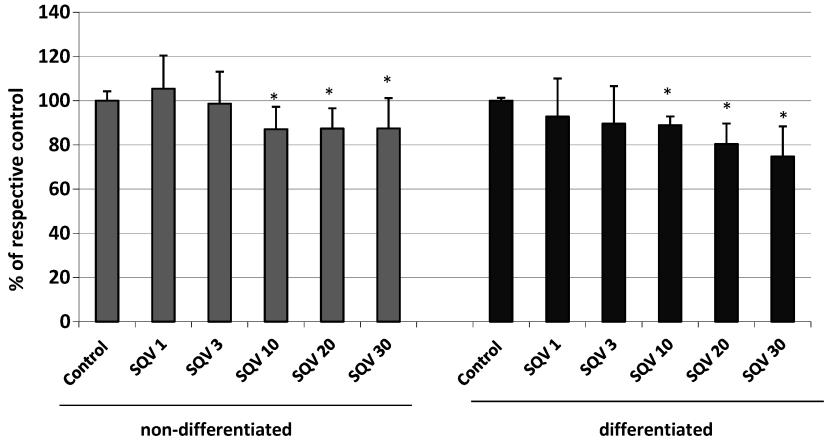

Fig. 5. Cellular ATP content in non-differentiated and differentiated Chub-S7 cells after 15 days incubation with different concentration of saquinavir (SQV, 1–30 μM). Data are presented as percentage of respective control. * $p < 0.01$ *vs.* respective control performed in five independent experiments in triplicate

(n = 5, p = 0.13), pointing to the insignificant lower content of ATP in differentiated adipocytes. Incubation with SQV (10–30 μM) significantly decreased the cellular ATP concentration, in both preadipocytes as well as mature adipocytes (Fig. 5). The above observations confirmed the observations that SQV induces mitochondrial dysfunction of pre- as well as adipocytes.

Influence of SQV on gene expression

The differentiation conditions caused a statistically significant inhibition of WNT signalling, and increased expression of genes involved in lipid droplet (LD) formation (Fig. 6).

SQV affected the differentiation-related gene expression such as: *WNT10a* – from the WNT-related tissue differentiation signalling [8]*;* transcription factors: *CEBP*-α [51]; endoplasmic reticulum stress (ER-stress) regulator – *ATF4* [56]; as well as PAT protein – *CIDEC* [58]; marker of differentiated adipocytes – adiponectin (*ADIPOQ*) [32]; and the TG synthesis/lipodystrophy gene expression regulator *LPIN1* [43].

The *WNT10a* gene expression was significantly down-regulated during differentiation of preadipocytes. This gene expression, was also decreased by SQV in non-differentiated cells. In differentiated cells SQV addition tended to activate expression of this gene, which may argue for inhibition of differentiation [8, 41] (Fig. 7).

The expression of *C/EBP*α was up-regulated during differentiation of preadipocytes, and this effect was inhibited by the presence of SQV (Fig. 7).

Differentiation conditions non-significantly affect the *ATF4* expression. SQV tended to down-regulate of *ATF4* expression in non-differentiated preadipo-

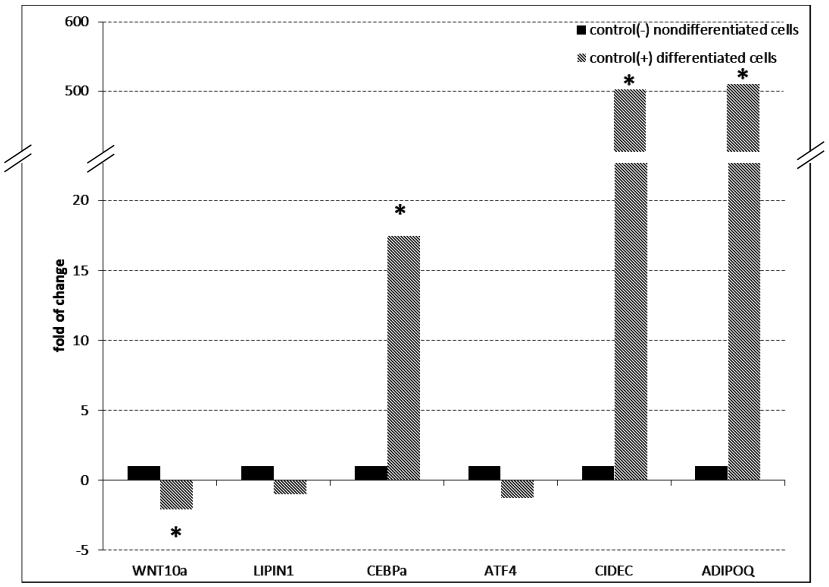

Fig. 6. Changes in relative gene expression of selected genes related to preadipocyte cells differentiation. Gene expression was measured using real-time PCR in non-differentiated and differentiated Chub-S7 cells. Data are presented as fold of change *vs.* control(–) non-differentiated cells. As a reference gene, expression of 18Sr-RNA was used. Significance: * $p < 0.02$ *vs.* control non-differentiated cells (performed in three independent experiments in triplicate

cytes but up-regulated expression of this gene in differentiated cells (Fig. 7).

As expected, differentiation of preadipocytes increased *ADIPOQ* gene expression. In preadipocytes SQV non significantly affected *ADIPOQ* expression. In adipocytes there was a non-significant tendency to lower its expression but only at higher concentrations of SQV (Fig. 7).

The *CIDEC* gene expression is related to lipid droplet formation in adipocytes and to the regulation of very-low density lipoprotein (VLDL) lipidation and secretion in hepatocytes [58]. *CIDEC* gene expression was significantly up-regulated during preadipocyte differentiation. This effect was decreased by the presence of SQV (Fig. 7).

The differentiation process did not significantly affect the lipodystrophy *LPIN1* gene expression [17]. Presence of SQV increased *LPIN1* expression in both undifferentiated and differentiated cells (Fig. 7).

Apoptosis and necrosis of preadipocytes incubated with SQV

SQV induced apoptosis and necrosis of the Chub-S7 cells. The effects were weaker in undifferentiated cells and observed only at the higher (> 10 μM) concentrations of SQV. In differentiated Chub-S7, the proapoptotic and pronecrotic effects of SQV were stronger, which suggested the greater susceptibility of mature adipocytes to these processes (Fig. 8).

Discussion

The presented study on pre- and differentiated Chub-S7 cells confirms the observations that SQV in concentrations found in patients during the AVT, significantly impairs the metabolic cell functions [28, 35, 44]. The differentiated adipocytes seems to be more sensitive to the SQV effects [28, 35].

The used differentiation conditions did not significantly change the mitochondrial membrane potential, oxygen consumption, as well as ATP production. Presence of SQV decreased most of these effects especially in differentiated cells.

Above metabolic effects were accompanied by the characteristic changes of the expression of genes related to activation of ER-stress (*ATF4*) [56], the differentiation process (*WNT10a, C/EBPα, ADIPOQ*) [8, 32, 51] and lipid droplet formation and apoptosis (*CIDEC*) [58], accompanying adipocyte differentiation [41].

Several groups of investigators documented that ER stress accompanies differentiation of preadipocytes and lipid droplet formation [1, 42]. It was confirmed in our study by changes in the *ATF4* gene expression. The role of *ATF4* in ER-stress and regulation of lipid droplet accumulation, metabolism and acceleration of apoptosis or even cellular death has been well documented [32, 51].

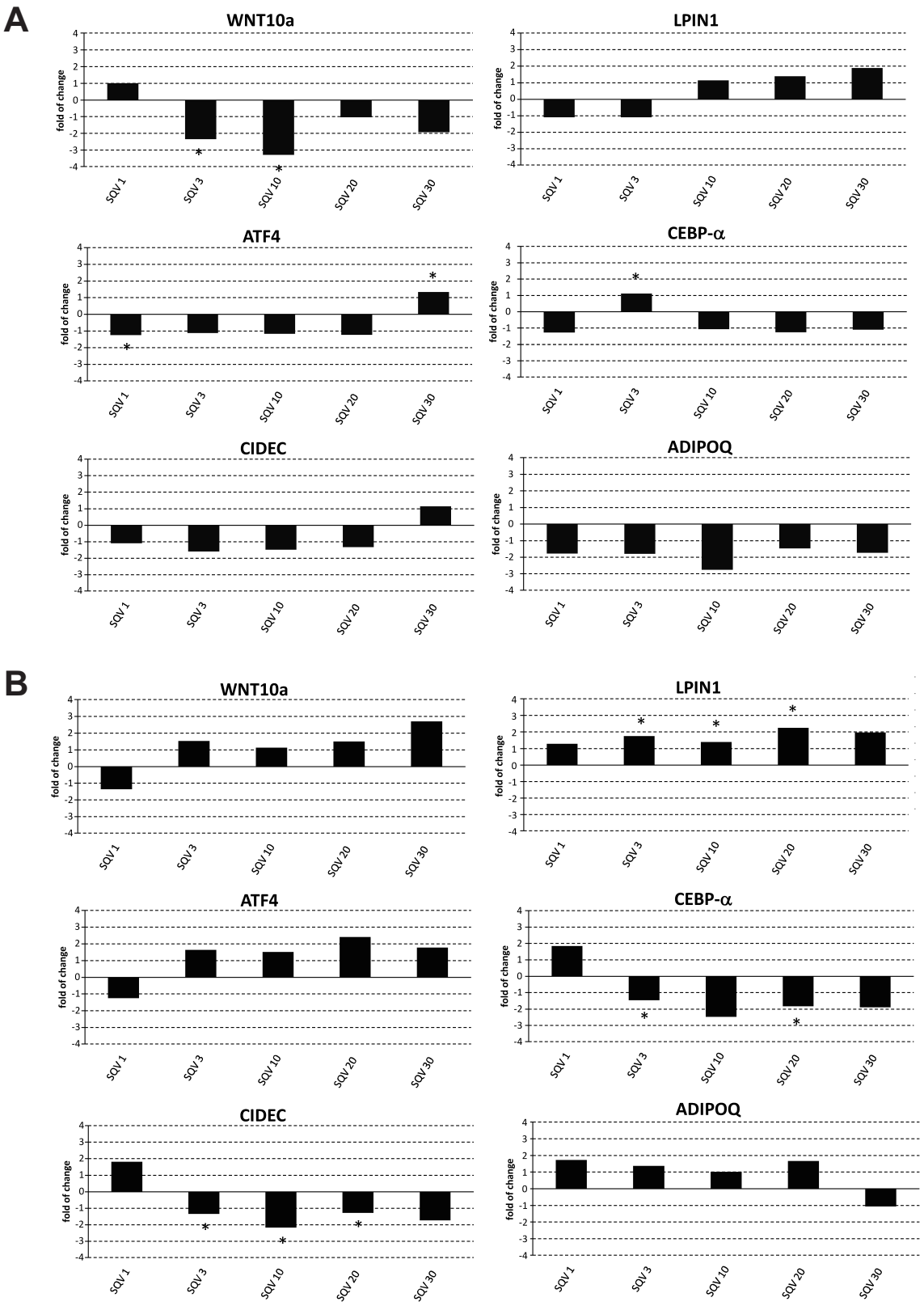

Fig. 7. Effect of SQV on the relative expression of selected related to preadipocyte cells differentiation genes. Gene expression was measured using real-time PCR in non-differentiated (**A**) and differentiated (**B**) Chub-S7 cells after 15 days of incubation with saquinavir (SQV, 1–30 μM). Data are presented as fold of change *vs.* respective control cells. As a reference gene, expression of 18SrRNA was used. Significance: * $p < 0.05$ *vs.* respective control cells performed in three independent experiments in triplicate

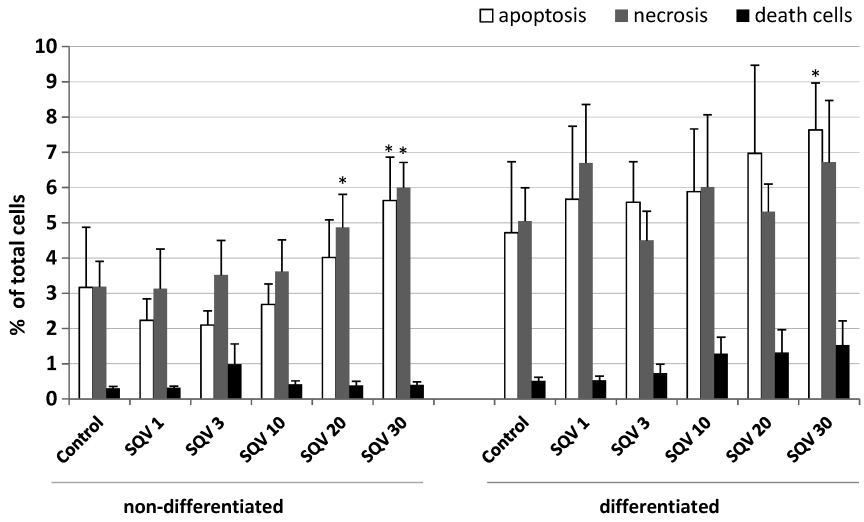

Fig. 8. Apoptosis and necrosis in non-differentiated and differentiated Chub-S7 cells after 15 days incubation with different concentration (1-30 μM) of saquinavir (SQV). Data are presented as percentage of total cells. * $p < 0.05$ *vs.* respective control performed in three independent experiments in triplicate

The observed up-regulation of adiponectin, confirmed the differentiation of preadipocytes [16, 29]. Adiponectin, secreted by adipose tissue, is responsible for insulin sensitivity, thus body metabolism, exerting cardioprotective, antineoplastic, anti-inflammatory, antiatherogenic and antiapoptotic properties. Decrease of this adipokine is characteristic for the obesity-related complications [16, 29, 33]. SQV did not significantly influence this gene expression, pointing, that it is not the main path of activity.

The *WNT10A* gene, a member of the Wnt inhibitory pathway of cell differentiation [8], was found to be down-regulated during development of white adipose tissue in *in vivo* and *in vitro* studies [8, 57]. In our experimental conditions the biphasic effect of SQV on *WNT10A* gene expression was observed – down-regulation during differentiation, but tendency to activation of this gene expression at higher concentrations of SQV. It may suggest, that inhibition of differentiation by high concentrations of SQV and promotion of undifferentiated cell phenotype characteristic for SQV what may result in development of lipodystrophy [8].

C/EBP-α (in cooperation with the other major adipogenesis-related transcription factors such as *SREBP-1, PPARγ,*) regulates the expression of key proteins related to adipocyte lipid content such as lipoprotein lipase, leptin and adiponectin and adipose tissue insulin sensitivity [9, 29, 30, 32, 51]. In previously published articles [30, 32, 51] as well as in our study, up-regulation of *C/EBP*α during preadipocytes differentiation was observed. This effect was inhibited by SQV in differentiated cells, arguing for mechanism of decrease of the adipocyte lipid content.

SQV also decreased expression of the *CIDEC* gene, coding the lipid droplet proteins, required for unilocular lipid droplet formation and optimal energy storage in human adipose tissue [48]. *CIDEC* gene expression may also promote apoptosis [58, 59]. Thus the observed changes in regulation of this gene expression may also participate in development of lipodystrophy.

LPIN1 is a bi-functional protein that dephosphorylates phosphatidic acid (PA) and acts in the nucleus as the co-activator of gene transcription responsible for the reduction of cellular triglyceride (TAG) generation and accumulation [32, 43]. Lipin-1 expression is regulated by nutritional status, insulin and other adipogenic factors such as adiponectin [17, 24, 43, 45]. During starvation, adrenalin (as well as fatty acids mobilized from adipose tissue) cause translocation of lipin-1 to ER and facilitate incorporation of FFAs into TAG [45]. Up-regulation of *LPIN1* expression is characteristic for loss of body fat, fatty liver development, hypertriglyceridemia and insulin resistance. Such effects cause *LPIN1* to be a candidate gene for human lipodystrophy [17, 24, 43]. The *LPIN1* gene expression was not significantly changed during differentiation of CHUB-S7 cells, but the addition of SQV up-regulate *LPIN1*, what may point to decrease of lipid droplet content, characteristic for SQV therapy-induced lipodystrophy.

Our results with the usage of the anti-HIV protease inhibitor SQV argue for its possible biphasic effect. Such effects may be related to the transient inhibition

or decrease of the endoplasmic reticulum (ER)-associated protein degradation (ERAD) system function [1, 10, 42]. Endoplasmic reticulum is the major nutrient sensor and signal transducing organelle that senses and responds to changes in cellular homeostasis [25, 46]. Excess of metabolic substrates or transient proteasome inhibition [42, 62] induces ER-stress, leading to lipid droplet formation [61]. SQV at low concentrations may exert such effect. Prolongation of proteasome dysfunction caused by higher concentrations of SQV during preadipocyte differentiation may lead to pathological effects resulting in cellular metabolic dysfunction (decrease of oxygen consumption, MMP value, ATP generation) and promotion of apoptosis/necrosis [19, 26]. The protease inhibitory effect of SQV seems to be nonspecific and may affect the proteasome activity as has been demonstrated by Toschi et al. [53]. Such cellular dysfunction may promote lipodystrophy in anti-HIV therapy with PI [1].

Impact of SQV on *AFT4* expression in differentiated cells, may point to an activation of apoptosis leading to lipodystrophy of adipose tissue. It has been demonstrated that *ATF4*-deficient mice are characterized by decreased expression of lipogenic genes in white adipose tissue, upregulation of expression of uncoupling protein 2 (UCP2) and increase in the free fatty acid β-oxidation-related genes. It is connected with lipolysis and increased energy expenditure [56]. It has been also shown, that ATF4 induces expression of the proapoptotic effector CHOP and could activate apoptosis [6].

Lipodystrophy as a side effect of anti-HIV therapy, was described two years after the introduction of PIs [4, 40, 49]. Treatment with ARVs is conductive to the development of lipodystrophy since the proteins encoded by HIV such as Nef-1 and Vpr interfere with the adipogenesis transcriptional factor PPARγ [21, 22].

Mitochondrial dysfunction is a significant element in the pathogenesis of lipodystrophy [21, 22, 54]. The presented study indicates that SQV, affects the mitochondrial function, connected with the decrease of oxygen consumption, mitochondrial membrane potential and ATP production. The effects were more pronounced in differentiated adipocytes, as compared with preadipocytes. This was paralleled by the presence of the higher percent of apoptotic and necrotic cells in the differentiated cell culture with SQV. Current reports indicate that some HIV infected patients, not treated with ARV drugs, demonstrate tissue specific decrease synthesis of mtDNA in peripheral blood mononuclear cells (PBMC) [18, 34]. Other authors did not observe differences in mtDNA content in adipose tissue and skeletal muscles under ARV treatment [34, 55].

It has been shown in several *in vitro* studies, as well as studies on animal models [2, 18] that different classes of ARVs such as NRTIs inhibit activity of polymerase-γ DNA, cause the mtDNA depletion and mitochondrial dysfunction [18, 21]. However, the mechanisms of the PIs impact on mitochondria is not completely understood. These drugs do not inhibit the DNA polymerase-γ and even increase the mtDNA expression [2]. Fragmentation of mitochondrial network, involving perinuclear clustering of mitochondria and Ca^{2+} accumulation inside these organelles by SQV was also reported [18, 47]. Mukhopadhyay et al. revealed that SQV as protease inhibitor affects mitochondrial processing protease [39].

Capel et al. postulated that PIs, such as, darunavir, atazanavir, lopinavir/ritonavir act through the distinct mechanisms to modify the function of adipocyte tissue, leading to lipodystrophy [7].

Our results point to the multiple activity of SQV leading to lipodystrophy. At lowest concentration stimulation of *CEBPα*, *CIDEC*, gene expression by SQV indicated even activation of genes related to differentiation. However, inhibition of the expression of these genes at the higher concentrations of the drug, corresponds to the loss of adipocyte lipid content pointing to phenotype change (dedifferentiation and lipodystrophy effects).

In conclusion, our results obtained on the cellular model of the SQV-induced lipodystrophy, suggest the multifactorial effects, related not only to impaired function of mitochondria, but also to the changes in the gene expression related to ER-stress, lipid droplet formation, preadipocyte differentiation. It may point to the polivalent changes in the nuclear proteins (such as of coactivators/corepressors of transcription) activity induced by PIs by concomitant modification of cellular proteases, which may result in iatrogenic lipodystrophy.

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