Cellular Physiology and Biochemistry
Cell Physiol Biochem 2016;39:1463-1470
DOI: 10.1159/000447849
Published online: September 09, 2016
Accepted: July 26, 2016

Original Paper

HIV Protease Inhibitors Apoptotic Effect in SH-SY5Y Neuronal Cell Line

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Key Words
Mother-to-child HIV transmission • Lopinavir • Ritonavir • Neuronal cell • Apoptosis • Mitochondrial damage

Abstract
Background: Prophylactic treatment regimens to prevent mother-to-child HIV transmission include protease inhibitors Lopinavir and Ritonavir. Lopinavir and Ritonavir have been reported to be able to induce intracellular oxidative stress in diverse cellular models, however scarce informations are available about protease inhibitor effects of in the central nervous system (CNS). In our study we evaluated the impact of protease inhibitors on a cell neuronal model. ***Methods:*** We treated a neuroblastoma cell line (SH-SY5Y) with increasing doses of Lopinavir and Ritonavir (0.1-1-10-25-50 µM), used alone or in combination, evaluating the impact of these drugs in terms of mitochondrial activity, with MTT cell proliferation assay; mRNA expression of heme oxygenase (HemeOH) and reactive oxygen species (ROS) levels with 2',7'-dichlorofluorescin diacetate (H2DCFDA) in order to assess oxidative stress; apoptotic cell death with flow cytometry. ***Results:*** We observed that Lopinavir and Ritonavir treatment, at 25 and/or 50 µM concentrations, induced mitochondrial damage, increase of heme oxygenase RNA expression levels and ROS generation, followed by apoptosis in SH-SY5Y. ***Conclusions:*** Our *in vitro* model demonstrates a damaging effect of HIV protease inhibitors on the neuroblastoma cell line, thus partially mimicking the impact of these drugs on the CNS of children born to HIV positive mothers undergone to antiretroviral treatment.

Introduction

The introduction of antiretroviral therapy (ART) in pregnant HIV positive women and newborns, contributed to strongly diminish HIV mother-to-child transmission (MTCT); however, side effects of ART in children, especially in the long term, are still scarcely known.

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HIV protease inhibitors (PIs), in particular Lopinavir and Ritonavir are currently used to prevent MTCT [1]; these drugs seem to have a good safety profile with minimal placental transfer, and the side effects during pregnancy in the mother (hepatotoxicity, effects on the hematic system, preeclampsia, gestational diabetes) and the child (prematurity, low birth weight, neonatal hyperbilirubinemia) are still to be elucidated [2].

An important issue, when considering HIV protease inhibitors possible side effects on children born to ART-treated HIV positive mothers, is the potential impact on the central nervous system (CNS), a structure still in development in the foetus and newborns exposed to ART.

So far, few studies have been reported on the neurodevelopment of uninfected children born to HIV-positive mothers treated with antiretroviral drugs, and children infected through HIV vertical transmission, born to mother who did not undergo antiretroviral treatment. Some authors observed mild neuropsychological deficits in HIV exposed uninfected children, particularly in the locomotor behavior, compared with healthy children; however, there is no clear quantitative evaluation of these defects as well as the correlation with the type of ART used [3, 4].

Neurologic side effects for Lopinavir and or Ritonavir have been described in mouse models [5, 6], while in humans protease inhibitors treatment has been reported as safe [7]. Moreover, protease inhibitor treatment, including Lopinavir and Ritonavir, has been related to cardiovascular complications and metabolic syndrome in HIV patients. Several studies, have reported activation of endoplasmatic reticulum stress after PIs treatment, apoptosis and an important increase in intracellular oxidative stress in cardiac myocytes and hepatocytes [8-10].

Therefore, different authors have reported oxidative stress as a key event in causing PIs adverse effects [11, 12]; however, few informations are available about the possible link between PIs and oxidative stress in the CNS. Pathological mechanisms have been observed in many neurological diseases, being mitochondrial dysfunction widely studied in the CNS. Apoptotic neuronal death is caused by dysfunctional mitochondria, a mechanism increased in different neurodegenerative disease and in normal cognitive impairment due to aging [13, 14]. Is it possible that the mild cognitive impairment observed in children treated with PIs could be due to dysfunctional mitochondria and increased oxydative stress?

All this considered, we tried to develop a simple cell model aimed at evaluating the possible impact of protease inhibitors on a neuronal cell line. We treated a neuroblastoma cell line (SH-SY5Y) with increasing doses of Lopinavir and Ritonavir, used alone or in combination, evaluating the influence of these drugs on apoptotic cell death, expression of heme oxygenase (HemeOH), ROS production and mitochondrial activity.

Materials and Methods

Cell Culture

SH-SY5Y neuroblastoma cells were chosen because widely used as model for various neurodegenerative diseases; SH-SY5Y also possesses different neuronal features.

SH-SY5Y, kindly provided by Prof. S. Gustincich (Department of Neurobiology, International School for Advanced Studied S.I.S.S.A.-I.S.A.S. Trieste, Italy) were cultured in 44.5% MEM/EBSS (Euroclone, Italy), 44.5% HAM'S/F12 (Euroclone, Italy), supplemented with 10% fetal bovine serum (FBS, Euroclone, Italy), non-essential amino acid solution 1× (NEAA, Euroclone, Italy), 2 mM glutamine and penicillin streptomycin amphotericin B 1× solution (Sigma Chemical Co. Aldrich St. Louis, MO) and used between passages 4 to 10 to avoid cellular senescence.

Once confluent, SH-SY5Y have been treated with increasing doses (0.1-1-10-25-50 μM) of Lopinavir, Ritonavir and the combination of the two drugs for 24 hours. The major concentrations of Lopinavir and Ritonavir (between 25 and 50 μM) have been chosen based on literature data [15, 16].

Mitochondrial activity

Mitochondrial activity (MA) was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Trevigen) [17]. Absorbance was measured in LT-400 Microplate Reader (Labtech, UK).

RNA isolation and real time-PCR

Total RNA was extracted from SH-SY5Y using the RNAqueous®-Micro Kit (Ambion®, Padova, Italy) and reverse transcribed into cDNA with a High Capacity cDNA Reverse Transcription Kit containing RNase Inhibitor (Applied Biosystems®, Monza, Italy). Semi-quantitative real-time PCR was performed using Taqman Gene Expression Assays for human ACTB (Hs99999903_m1) and HemeOH (Hs01110250_m1) genes (Applied Biosystems®, Monza, Italy) with the Applied Biosystems 7500 Fast Real-Time PCR System. PCR amplification cycle was as follows: after denaturation at 96°C for 10 min, 40 PCR cycles were performed composed by 15 sec at 95°C and a final melting step for 1 min at 60°C. All samples were analyzed in triplicate (for further details see Tricarico et al. [18]).

ROS measurement

To measure cellular ROS, we used the molecular probe 2',7'-dichlorofluorescin diacetate (H2DCFDA) (Sigma Aldrich, Saint Louis, Missouri, United States). SH-SY5Y cells were loaded with 10 μM H2DCFDA for 30 min. Fluorescence was measured employing the fluorometer GloMax®-Multi Detection System (Promega, Fitchburg, Wisconsin, United States). Wavelengths of excitation and emission were 490 and 510-570nm, respectively. Fluorescence intensity was normalized to % of cell alive, estimated with (MTT) cell proliferation assay (Trevigen).

Apoptosis analysis

Apoptosis, programmed cell death (PCD), has been evaluated in SH-SY5Y cells with flow cytometry (Annexin V-FITC Apoptosis Detection Kit, Immunostep, Spain). Fluorescence was acquired with CyAn ADP analyzer and Summit software (Beckman Coulter, Brea, CA, USA), then analysed with FlowJo software (version 7.6, Treestar, Inc., St Ashland, OR, USA).

Data analysis

Data analyses were performed with one-way ANOVA and Bonferroni correction comparing not treated cells (NT) with other experimental conditions. Statistical analysis has been performed using GraphPad Prism software (version 5.0, GraphPad Software, Inc., La Jolla, CA, USA).

Results

Treatment with increasing doses of either Lopinavir or Ritonavir, alone and in combination, induces a decrease in mitochondrial activity (MA) (Fig. 1A,B,C). Lopinavir

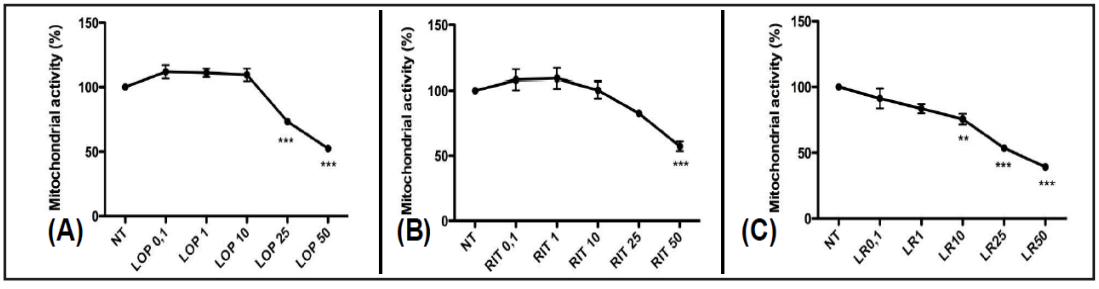

Fig. 1. Lopinavir and Ritonavir induced mitochondrial damage in SH-SY5Y. SH-SY5Y cells were treated with increasing doses (0.1, 1, 10, 25, 50 μM) of Lopinavir (A), Ritonavir (B) and the combination (C) of the two drugs for 24 hours. Mitochondrial activity (MA) was evaluated by MTT assay. Data are shown as the percentage of levels compared with NT, considered as 100% of mitochondrial activity in the respective experimental setting. Data analyses were performed with one-way ANOVA and Bonferroni correction comparing NT (not treated) cells with the other experimental conditions; ** $p < 0.01$; *** $p < 0.001$.

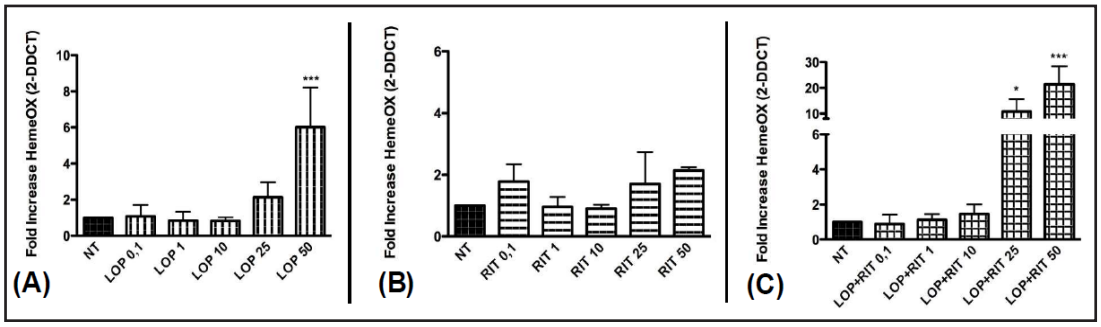

Fig. 2. Lopinavir and Ritonavir induced increase in HemeOX expression. SH-SY5Y cells were treated with increasing doses (0.1, 1, 10, 25, 50 μM) of Lopinavir (A), Ritonavir (B) and the combination (C) of the two drugs for 24 hours. Expression of HemeOX was measured after drugs treatment. Analyses were performed using real time quantitative polymerase chain reaction (PCR), and results were normalized to ACTB expression. Expression of untreated cells was normalized to 1. Expression data for the three experiments are reported as 2−ΔΔCt average ± SD, in which ΔΔCt = ΔCt_stimulated HC−ΔCt_RHC. Data analyses were performed with one-way ANOVA and Bonferroni correction comparing NT (not treated) cells with the other experimental conditions; * $p < 0.05$; *** $p < 0.001$.

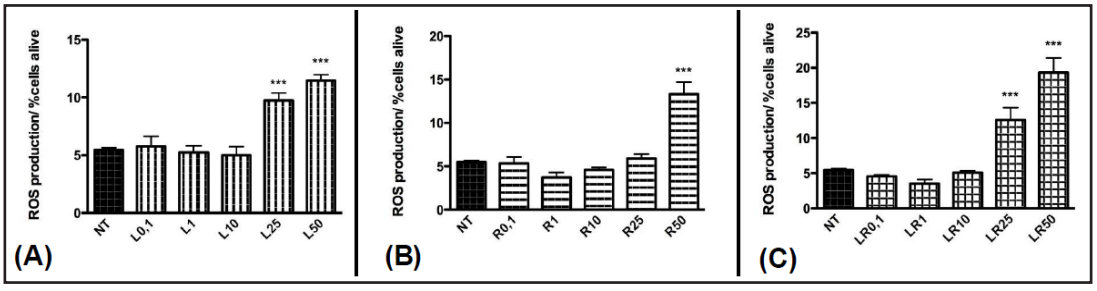

Fig. 3. Lopinavir and Ritonavir induced increase in ROS production. SH-SY5Y cells were treated with increasing doses (0.1, 1, 10, 25, 50 μM) of Lopinavir (A), Ritonavir (B) and the combination (C) of the two drugs for 24 hours. ROS production was measured after drugs treatment. Analyses were performed using 10 μM H2DCFDA. Fluorescence intensity was normalized to % of cell alive subsequently estimated with MTT cell proliferation assay. Data analyses were performed with one-way ANOVA and Bonferroni correction comparing NT (not treated) cells with the other experimental conditions; *** $p < 0.001$.

prompts a significant decrease of mitochondrial activity only at the higher concentrations (MA values, 25 μM: 73,35±2,11, p<0,001; 50 μM: 52,42±5,55, p<0,001; NT: 100) (Fig. 1A), while only after 50 μM Ritonavir treatment, it was possible to observe a significant decrease in mitochondrial dehydrogenase enzyme activity (MA values, 50 μM: 57,37±8,48, p<0,001; NT: 100) (Fig. 1B). Co-treatment with Lopinavir and Ritonavir causes a significant decrease of mitochondrial activity at lower concentrations: 10, 25 and 50 μM treatment (MA values, 10 μM: 75.51±9,16, p<0,01; 25 μM: 53,59±4,69, p<0,001; 50 μM: 39,25±2,22, p<0,001; NT: 100) (Fig. 1C).

These results suggest that Lopinavir and Ritonavir treatment impacts and impairs mitochondrial activity. For this reason, we decided to analyze HemeOX expression levels in SH-SY5Y cell line. HemeOX is one of the key players in protective mechanisms against oxidative stress, being its expression modulated by oxidative or chemical stress. The increase of HemeOX has been demonstrated as triggering mitochondrial dysfunction and cellular pathology [19, 20].

Lopinavir treatment increased HemeOX expression was observed only with 50 μM treatment (Fold increase HemeOX values, 50 μM: 6,02±2,18, p<0,001; NT: 1) (Fig. 2A), while no significant variation in HemeOX expression levels was observed after Ritonavir treatment (Fig. 2B). However co-treatment with Lopinavir and Ritonavir significantly increased

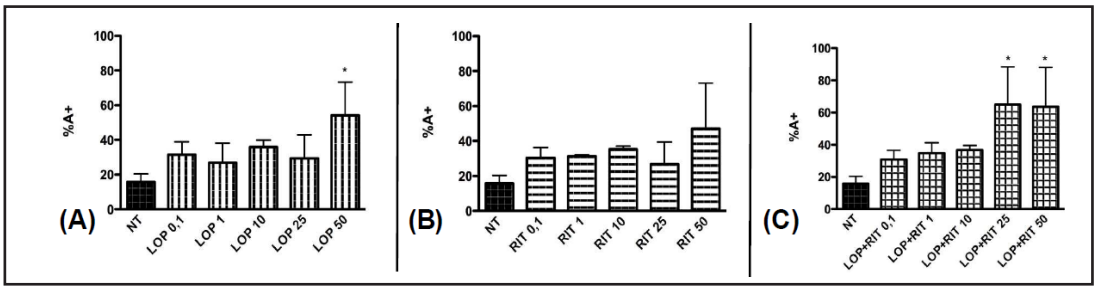

Fig. 4. Lopinavir and Ritonavir induced apoptosis in SH-SY5Y. SH-SY5Y cells were treated with increasing doses (0.1, 1, 10, 25, 50 μM) of Lopinavir (A), Ritonavir (B) and the combination (C) of the two drugs for 24 hours. A%: bars represent the mean % of Annexin V positive cells (A+) ± standard deviation (SD) of three independent experiments. Data analyses were performed with one-way ANOVA and Bonferroni correction comparing NT (not treated) cells with other experimental conditions; * $p < 0.05$.

HemeOx expression at 25 and 50 μM concentrations (Fold increase HemeOX values, 25 μM: 10,80±4,79, p<0,05; 50 μM: 21,39±7,06, p<0,001; NT: 1) (Fig. 2C).

To confirm the increase of oxidative stress we measured ROS generation after treatment with Lopinavir and Ritonavir alone and in combination. In accordance with the results of HemeOX expression levels, Lopinavir treatment induced a significant increase of ROS production at 25 and 50 μM concentration (ROS production/%cell alive, 25 μM: 9,74±0,65, p<0,001; 50 μM: 11,46±0,50, p<0,001; NT: 5,46±0,18) (Fig. 3A), while Ritonavir treatment induces an increase of ROS production only at the higher concentrations (ROS production/%cell alive, 50 μM: 13,3±1,37, p<0,001; NT: 5,46±0,18) (Fig. 3B). Co-treatment with Lopinavir and Ritonavir significantly increased ROS production at 25 and 50 μM concentrations (ROS production/ %cell alive, 25 μM: 12,57±1,77, p<0,001; 50 μM: 19,33±2,09, p<0,001; NT: 5,46±0,18 (Fig. 3C).

Then we assessed apoptosis in our neuronal cell model, to determine if the mitochondrial impairment followed by oxidative stress was able to increase cell death. Lopinavir treatment in SH-SY5Y induced augmented apoptosis, compared with untreated cells, only at the highest concentration (A+% values, 50 μM: 54,10±19,23, p<0,05; NT: 15,80±4,57) (Fig. 4A), while Ritonavir treatment resulted in increased apoptosis for all tested concentrations, even if the difference with the untreated condition was not statistically significant. (Fig. 4B). Administration of the two drugs in combination provoked a significant increase of apoptotic cells, with respect to untreated cells, at concentrations of 25 and 50 μM (PCD values, 25 μM: 64,97±23,64, p<0,05; 50 μM: 63,63±24,43, p<0,05; NT: 15,80±4,57) (Fig 4C).

Discussion

Our results show that Lopinavir and Ritonavir treatment at 25 and/or 50 μM concentration in SH-SY5Y neuronal cell line induces increase of apoptosis, HemeOX expression levels and ROS production. HemeOX is one of the key players acting as protective mechanisms against oxidative stress; so its increase indicates a raise of oxidative stress, confirmed by the augmented H2DCFDA fluorescence.. So, the increased apoptosis observed after treatment with Lopinavir/Ritonavir could be due to the oxidative stress following mitochondrial damage. Supporting evidences are the results obtained with MTT assay showing an important decrease in mitochondrial activity even at concentrations (10μM) not inducing cell death, HemeOx increase and ROS production.

Moreover, Deng W. and coauthors reported augmented oxidative stress connected to mitochondrial damage, after Lopinavir and Ritonavir treatment in cardiac myocytes [11]. Another research group identified Lopinavir as a potent oxidative stress inducer, which causes ER stress, a common ART-induced side effect, in hepatocytes and in intestinal epithelial cells [12]. Instead, in erythrocytes from healthy volunteers Lopinavir treatment

enhanced eryptosis, correlated again with oxidative stress [21]. Therefore, ART-treatment seems to increase oxidative stress in many different types of cellular models; unfortunately, the molecular mechanism underlying this effect is not yet fully elucidated and little is known about the relationship between oxidative stress and ART-treatment in CNS. For these reasons further studies are essential to understand this strong correlation between oxidative stress and ART-treatment.

In this study we observed a damaging impact of Lopinavir and Ritonavir on SH-SY5Y neuronal cell line; these findings could lead us to hypothesize possible neurologic side-effects on CNS in children born to HIV positive mothers, treated with HIV proteinase inhibitors. However this is just a preliminary conjecture, since we have not considered the influence of virus infection on apoptosis and mitochondrial impairment; we are also aware that we used a neuroblastoma cell line model, which partially mimic CNS. Moreover, we have to consider that the drugs concentrations used in our experiments, even if chosen based on previously reported literature data, could not precisely reflect the administration conditions in newborns [15, 16]. Nevertheless it should be said that HIV protease inhibitors have poor penetration into the cerebrospinal fluid (CSF) [22]; on the other hand, the blood brain barrier (BBB) in the embryo, foetus, and newborn is immature and thus fragile, making the developing brain more vulnerable to drugs [23]; furthermore, in many cases of HIV infection the infiltration of infected macrophages and lymphocytes into the brain across the vascular boundary perturbs the BBB, therefore, the ability to cross the physiological BBB is not necessarily correlated with the administered amount of drugs in the CNS [24].

Our hypothesis suggesting a possible involvement of Lopinavir/Ritonavir in the neurologic outcome of children born to HIV mothers treated with protease inhibitors should be corroborated (or not) by clinical observations; however at present time studies concerning this topic are still limited in number and report just partial findings. As an example Spaulding et al. recently described the neurologic effects of ART in 1400 children born to HIV positive mothers from Latin America and the Caribbean (Brazil, Argentina, Peru, Mexico, Bahamas, and Jamaica). The authors reported augmented prevalence of neurologic disorder (9.6%), mostly microcephaly (7.5%), possibly related to ART treatment; however within the antiretroviral drugs used, namely 3TC (98.1%), ZDV (94.2%), NFV (40.6%) and Nevirapine (35.9%), the combination of Lopinavir/Ritonavir was the less administrated to mothers and children. Even if of interest for the great number of mothers and children considered and the clinical observations, this study did not show a clear correlation between Lopinavir/Ritonavir administration and neurologic defects, consequently not providing useful information about the safeness and clinical consequences of these drugs in children born to HIV positive mothers [4].

So, considering the lack of clinical studies clearly demonstrating the effects of protease inhibitors in children born to HIV positive mothers, our SH-SY5Y neuronal cell line model could contribute to improve knowledge of the direct effect of Lopinavir/Ritonavir, the most commonly used HIV protease inhibitors to prevent MTCT, on the CNS of children born to HIV positive mothers undergoing ART treatment.

Conclusions

We demonstrated that 25 and/or 50 μM concentrations of Lopinavir/Ritonavir administered to SH-SY5Y neuronal cell line are associated with an increase of cell death through apoptosis and mitochondrial impairment (also observed at 10 μM concentration) with augmented oxidative stress and ROS production, opening new questions about the neurological side effects of these drugs widely used in the prevention of MTCT.

Acknowledgements

This work was supported by grants from the Institute for Maternal and Child Health IRCCS "Burlo Garofolo" (RC 42/11; RC07/08).

Disclosure Statement

There are no conflicts of interest.

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