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MOLECULAR MECHANISMS OF HIV TAT-INDUCED NEURONAL TOXICITY: INVESTIGATING THE ROLE OF POLYAMINE OXIDASES AS MEDIATORS OF ROS PRODUCTION AND THE ACTIVATION OF THE ANTIOXIDANT CELL RESPONSE

MECCANISMI MOLECOLARI COINVOLTI NELL'EFFETTO NEUROTOSSICO DELLA PROTEINA TAT DEL VIRUS HIV: VALUTAZIONE DEL RUOLO DELLE POLIAMMINE OSSIDASI, COME MEDIATORI DELLA PRODUZIONE DI ROS, E STUDIO DELL'ATTIVAZIONE DELLA RISPOSTA CELLULARE ALLO STRESS OSSIDATIVO

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ABSTRACT

Chronic oxidative stress plays an important role in the pathogenesis of HIV-associated dementia (HAD). Like many viruses, HIV-1 initiates oxidative stress in infected cells. However, HIV-1-derived proteins can also induce oxidative stress in uninfected cell types (e.g., neurons, endothelia and astrocytes), through the release of cytotoxic factors, including nitric oxide (NO) and reactive oxygen species (ROS) (Mattson *et al*., 2005). These two molecules generates peroxinitrite, the main cause of a chronic oxidative/nitrosative stress often observed in the pathogenesis of many neurodegenerative diseases, including HAD (Allan Butterfield *et al.*, 2006; Steiner *et al.*, 2006). Moreover, HIV proteins such as gp120 and Tat are able to activate cell surface receptors such as the N-methyl-D-aspartate receptor (NMDAR) leading to excitotoxicity (Mattson *et al*., 2005). Literature data indicated that HIV-Tat is an important neurotoxic effector (Eliseo *et al*., 2007) being able to activate NMDAR and induce apoptotic neuronal death. In particular, the stimulation of the polyamine-sensitive type of NMDAR is associated with the neurotoxic effects induced by HIV-Tat (Li *et al*., 2008). It should be reminded that Tat-induced NMDAR activation stimulate NO production in astrocytes and neuronal cultures (Eliseo *et al*., 2007). Polyamines are known to be essential for normal cell growth and differentiation, but their oxidative products may act as negative regulators of cell growth and survival. Indeed, many evidences link the products of polyamine degradation to excitotoxicity (Wood *et al.*, 2006) and neurodegeneration (Wood *et al*., 2007). We hypothesize that HIV-1-Tat may induce oxidative stress and cell death through the production of both NO and H_2O_2, the latter by a mechanism involving polyamine oxidation.

A chronic oxidative/nitrosative stress occurs in cells when the production of oxidants exceeds the intracellular antioxidant defences. In the cell, a fundamental antioxidant defence system is represented by the induction of cytoprotective (phase-2) enzymes, such as Glutathione transferases (GST), Glutamate cysteine lygase (GCL), and Heme oxygenase-1 (HO-1). Under basal conditions, these enzymes are present as a fraction of their full capacity, but the transcription of their cognate genes can be co-ordinately upregulated by exposure to a variety of stimuli, including oxidants, through the activation of a *cis*-acting enhancer termed antioxidant response element (ARE). ARE-mediated gene activation is coordinated by the Nrf-2 transcription factor, which is considered a major player orchestrating the antioxidant defence response in the cell. It has been recently reported that Nrf2 can interfere with the activity of Tat in inducing HIV-1 LTR transactivation (Hong-Sheng *et al*., 2009). Thus, we hypothesize that Tat

may directly affect Nrf2-dependent gene expression, likely preventing the neuronal antioxidant cell response (Jeffrey *et al*., 2007; de Vries *et al*., 2008).

This project was aimed at clarifying the molecular mechanisms of HIV-Tat-induced neuronal toxicity, focusing on the role of polyamine metabolism in Tat-induced oxidative stress and on the activation of the antioxidant response induced by HIV-Tat in astroglial and neuronal cells. The experiments were performed on human astrocytoma (U373-MG), neuroblastoma (SH-SY5Y) and mouse neuroblastoma (NIE-115) cell lines.

In the first step of the study we carried out experiments to evaluate the role of polyamine oxidases as mediators of oxidative stress in cells treated with HIV-Tat recombinant protein. We found that Tat was able to induce ROS production mainly in neuroblastoma rather than in glioma cells. Accordingly, Tat affected cell viability of only neuronal cells. Astrocytoma cells, indeed appeared completely resistant to the cytotoxic effect of Tat. To evaluate the involvement of polyamine metabolism in the neurotoxicity elicited by Tat we measured ROS content and cell viability in Tat-stimulated cells in absence or presence of the polyamine oxidases inhibitors Chlorhexidine (CHX), a structural spermine analogue, affecting the enzymatic activity of SMO (*spermine oxidase*) and APAO (*N1-acetyl polyamine oxidase*). The results showed that CHX inhibited ROS formation and restored cell viability, suggesting that polyamine degradation products such as H_2O_2 may be involved in neuronal cell death induced by Tat. In addition, by RT-PCR we evaluated the gene expression of the enzymes involved in polyamine catabolism (SMO, APAO, ODC and SSAT), after treating SH-SY5Y with Tat. We found that mRNA levels of enzymes SMO, APAO and SSAT were up-regulated and that SMO activity was higher in HIV-Tat stimulated SH-SY5Y cells, respect to the untreated cells. Next, to assess whether the activation of NMDA receptor might play a role in triggering the up-regulation of polyamine metabolism we performed experiments with the NMDA receptor antagonist MK-801. We observed that MK-801 pre-treatment of Tat-stimulated cells prevented ROS generation and restored cell viability. Moreover, the NMDA-dependent ROS generation was abolished by CHX pretreatment in SH-SY5Y cells. As the neurotoxic effect of HIV-Tat is likely due to the overstimulation of NMDA receptor and concomitant nitric oxide increasing, we tested the effect of the NOS (Nitric Oxidase Sinthase) inhibitor L-NAME on the survival of SH-SY5Y cells treated with Tat, demonstrating that L-NAME pre-treatment restored the viability of Tat-treated cells. Our results strongly suggest that the origin of ROS generation could be related to

spermine/spermidine metabolism. We provided also clearly evidence of the involvement of polyamine-derived hydrogen peroxide in Tat induced neuronal cell death since the specific inhibition of SMO/APAO completely restored cell viability. In regard to the involvement of the NMDA receptor in this pathway we demonstrated that inhibiting polyamine metabolism by specific inhibitor completely prevented NMDA-induced cell death as well as ROS production.

HIV is known to progressively deplete GSH content in patients. In our cell culture models we found that Tat treatment caused a significant decrease of GSH levels only in neuroblastoma cells, evidencing an increase of oxidative stress in neurons. Thus in the second step of the project, we analyzed the activation of the antioxidant Nrf2/ARE pathway. In particular TransAM assays (DNA-binding ELISA) has been performed to evaluate Nrf2 transcription factor activation. In this respect, the treatment with Tat was able to activate Nrf2 in both cell lines. Further, we analyzed the mRNA expression of the ARE-genes coding for HO-1 and GCL after Tat treatment, by RT-PCR. The results underlined the up-regulation of HO-1 and GCL-C mRNA levels in U373-MG, indicating that the activation of the antioxidant response occurred in astrocytes, but not in neurons. Finally, co-immunoprecipitation experiments indicated a physical interaction between Nrf2 and Tat proteins occurred almost exclusively in SH-SY5Y.

Our findings clearly show the inability of neuroblastoma cells to activate an efficient antioxidant response even in the presence of activated Nrf2.

Moreover, our preliminary data indicate a different ability of astroglial and neuronal cell lines in releasing Tat. This could result in the accumulation of the viral protein within neuronal cells thus increasing its toxic effect, likely interfering with the cell antioxidant response machinery.

On the contrary astrocytes, being able to release the viral protein into the extracellular space, can activate the antioxidant pro-survival response. In conclusion, the obtained results suggest a mechanism by which Tat could modulate the antioxidant response in neurons thus leading to cell death.

However, additional studies will be required to obtain a detailed understanding of the specific proteins involved in regulation of this pathway.

SINTESI

Lo stress ossidativo cronico riveste un ruolo importante nella patogenesi della demenza associata all'infezione da HIV (HAD). Questa patologia neurodegenerativa colpisce circa il 30% dei pazienti sieropositivi adulti. Il virus HIV-1 può causare stress ossidativo, anche nelle cellule non infettate (neuroni, cellule endoteliali e astrociti), attraverso il rilascio di diversi fattori neurotossici quali l'ossido di azoto (NO) e specie reattive dell'ossigeno (ROS) (Mattson et al., 2005). Queste due molecole generano perossinitrito, la principale causa di stress ossidativo/nitrosativo cronico spesso osservata nella patogenesi di molte malattie neurodegenerative, tra le quali la HAD (Allan Butterfield et al., 2006; Steiner et al., 2006). Inoltre, le proteine dell'HIV-1 gp120 e Tat possono attivare recettori di membrana come l'NMDA (recettore del glutammato di tipo N-metil-D-aspartato) e causare eccito-tossicità (Mattson et al., 2005). In particolare è stato evidenziato in letteratura che la proteina Tat provoca i suoi effetti neurotossici interagendo con un sito specifico per le poliammine sul recettore NMDA (Li et al., 2008). Va inoltre ricordato che l'ipersimolazione del recettore NMDA da parte di Tat stimola la produzione di NO in colture cellulari di astrociti e neuroni (Eliseo et al., 2007).

Le poliammine sono note per essere necessarie alla crescita e al differenziamento cellulare, ma i loro prodotti di ossidazione possono invece agire come regolatori negativi di queste funzioni. Infatti, numerose evidenze sperimentali (Wood et al., 2007), attribuiscono ai prodotti del catabolismo delle poliammine una funzione neurotossica (Wood et al., 2006). La nostra ipotesi è che Tat possa indurre stress ossidativo e morte cellulare attraverso la produzione di NO e perossido di idrogeno, quest'ultimo generato attraverso un meccanismo che coinvolge l'ossidazione delle poliammine. Lo stress ossidativo/nitrosativo cronico si verifica quando nelle cellule la produzione di specie ossidanti prevale sui meccanismi di difesa della risposta antiossidante. Nella cellula un sistema fondamentale di difesa antiossidante è rappresentato dall'induzione di enzimi citoprotettivi detti "enzimi di fase-2", come glutathione S-transferase (GST), la glutammato cisteina ligasi (GCL) e l'eme-ossigenasi-1 (HO-1). In condizioni basali questi enzimi sono espressi solo in minima parte, ma la trascrizione dei loro geni può essere immediatamente incrementata dall'esposizione ad una varietà di stimoli, primi tra tutti quelli di natura pro-ossidante, grazie alla presenza di un elemento di risposta antiossidante (ARE). L'attivazione dei geni mediata dalla sequenze ARE è coordinata dal fattore di trascrizione Nrf2, considerato forse il più importante effettore della risposta antiossidante nella cellula. È stato riportato in letteratura che l'attivazione

della via di segnalazione mediata da Nrf2 negli astrociti è sufficiente a conferire protezione contro lo stress ossidativo e tale effetto si estende anche alla maggior parte dei neuroni corticali (Kraft et al., 2004). Recentemente è stato dimostrato che Nrf2 può interferire con l'attività trans-attivante di Tat (Hong-Sheng et al., 2009). La nostra ipotesi è che Tat possa interagire direttamente con Nrf2 interferendo con l'espressione dei geni da esso regolati, prevenendo in tal modo la risposta cellulare neuronale allo stress ossidativo (Jeffrey et al., 2007; de Vries et al., 2008.

Questo progetto di ricerca ha dunque lo scopo di chiarire i meccanismi molecolari che sono alla base della neurotossicità indotta da Tat, con particolare interesse rivolto al ruolo del metabolismo delle poliammine nell'induzione dello stress ossidativo da parte della proteina virale. Un ulteriore obiettivo della ricerca è l'approfondimento delle conoscenze sui meccanismi di attivazione della risposta antiossidante nelle cellule astrogliali e neuronali. Gli esperimenti sono stati condotti su linee cellulari umane, di origine astrogliale (U373-MG), neuronale (SH-SY5Y) e su una linea di neuroblastoma murino (NIE-115).

Nella prima fase del nostro studio abbiamo valutato il ruolo delle poliammino-ossidasi come mediatori di stress ossidativo nelle cellule trattate con la proteina ricombinante Tat. I risultati ottenuti hanno dimostrato che Tat può incrementare la produzione di ROS soprattutto nelle cellule di neuroblastoma, piuttosto che negli astrociti. Conseguentemente, è stata osservata la riduzione della vitalità cellulare indotta da Tat esclusivamente nelle cellule neuronali. Infatti, le cellule di astrocitoma sono risultate resistenti agli effetti citotossici della proteina virale.

Per valutare il coinvolgimento del metabolismo delle poliammine nell'induzione della neurotossicità, abbiamo misurato il contenuto di ROS e la vitalità delle cellule esposte a Tat in presenza ed in assenza di Clorexidina (CHX), un inibitore specifico delle poliammino-ossidasi, analogo strutturale della spermina, che influenza l'attività enzimatica di SMO (spermine oxidase) e APAO (N1-acetyl polyamine oxidase). I risultati ottenuti hanno evidenziato che la CHX è in grado di inibire la formazione di ROS e di ripristinare la vitalità delle cellule stimolate con Tat, suggerendo che i prodotti di degradazione delle poliammine, come il perossido di idrogeno, potrebbero essere coinvolti nella morte neuronale. Inoltre, nelle cellule SH-SY5Y trattate con Tat abbiamo studiato, mediante RT-PCR, l'espressione genica degli enzimi coinvolti nel catabolismo delle poliammine (SMO, APAO, ODC e SSAT). Abbiamo dimostrato che nelle cellule SH-SY5Y trattate con Tat i livelli degli mRNA degli enzimi SMO e

APAO (coinvolti nella regolazione del metabolismo della spermina/spermi dina) risultavano aumentati come anche l'attività enzimatica di SMO.
Successivamente, è stato verificato il coinvolgimento del recettore NMDA in questo meccanismo attraverso l'uso dell'antagonista specifico MK-801. In particolare, è stato osservato che il pre-trattamento con l'MK-801 previene la formazione dei ROS e ripristina la vitalità delle cellule stimolate con Tat. Inoltre, la produzione di ROS indotta da NMDA veniva inibita dalla CHX indicando il coinvolgimento del metabolismo delle poliammide in questo meccanismo. L'effetto neurotossico di Tat è anche legato all'aumento dei livelli di NO, infatti l'inibitore delle NOS (Sintetasi dell'Ossido Nitrico), L-NAME, riduce la mortalità delle cellule di neuroblastoma. I nostri risultati suggeriscono con forza che la produzione di ROS può avere origine dal metabolismo della spermina/spermidina. Abbiamo anche fornito una chiara dimostrazione del coinvolgimento del perossido di idrogeno derivato dalle poliammine nella morte neuronale indotta da Tat, dato che l'inibizione specifica di SMO e APAO era in grado di ripristinare completamente la vitalità cellulare.
Lo stress ossidativo viene anche definito come una condizione di sbilanciamento, tra i meccanismi cellulari anti-ossidanti (es. la regolazione del GSH intracellulare e della cisteina) a favore di quelli pro-ossidanti (es. la produzione di ROS). E' noto dalla letteratura che da pazienti con infezione da HIV vanno incontro ad una graduale riduzione dei livelli di GSH. Abbiamo riscontrato che nei nostri modelli cellulari il trattamento con Tat è associato ad una significativa riduzione dei livelli di GSH solo nelle cellule di neuroblastoma, evidenziando l'aumento di stress ossidativo nei neuroni.
Nella seconda fase del progetto è stata valutata, l'attivazione del pathway anti-ossidante Nrf2/ARE. In particolare, sono state condotte analisi mediante saggio TransAM, per analizzare lo stato di attivazione del fattore di trascrizione Nrf2. È stato osservato che il trattamento con Tat ha indotto un aumento dell'attività di Nrf2, con la conseguente migrazione nucleare del fattore in entrambe le linee cellulari. Mediante RT-PCR sono stati analizzati nelle cellule trattate con Tat, i livelli di espressione dei geni ARE-dipendenti codificanti per gli enzimi di "fase-2" HO-1e GCL. I risultati hanno evidenziato l'innalzamento dei livelli dei messaggeri dell'HO-1 e della subunità catalitica di GCL esclusivamente nelle cellule U373-MG, indicando che l'attivazione della risposta anti-ossidante avveniva negli astrociti, ma non nei neuroni. Infine, gli esperimenti di co-immunoprecipitazione hanno rivelato una probabile interazione fisica tra le due proteine Tat e Nrf2, pressochè esclusivamente nelle cellule di

neuroblastoma. I nostri risultati mostrano chiaramente l'incapacità delle cellule di neuroblastoma di attivare una efficace risposta antiossidante, nonostante il fattore di trascrizione Nrf2 sia attivato anche in queste cellule. Inoltre, i nostri dati preliminari indicano una differente capacità delle cellule astro gliali rispetto a quelle neuronali di rilasciare Tat nel sopranatante di coltura. Questo porterebbe all'accumulo della proteina virale all'interno delle cellule neuronali con il conseguente aumento dell'effetto citotossico, dovuto anche alla compromissione dei meccanismi che regolano la risposta anti-ossidante. Al contrario, gli astrociti, essendo in grado di rilasciare la proteina virale nello spazio extra-cellulare, possono attivare la risposta anti-ossidante per la sopravvivenza. In conclusione, i risultati ottenuti suggeriscono un meccanismo mediante il quale Tat potrebbe compromettere la risposta anti-ossidante dei neuroni inducendo così la morte cellulare. Tuttavia sono necessari ulteriori studi per arrivare ad una comprensione più approfondita dei meccanismi e delle proteine coinvolte in questi processi.

1. INTRODUCTION

1.1. Oxidative stress and Neurodegenerative Disorders

Oxidative stress can be defined as an imbalance between the production of free radicals and the antioxidant cell systems, with the shift towards free radicals generation. Reactive Oxygen/Nitrogen Species (ROS/RNS) such as hydroxyl radical, peroxyl radical, superoxide anion, hydrogen peroxide and peroxynitrite are highly reactive, toxic oxygen moieties (fig.1). The half-life of ROS or RNS species varies from nanoseconds for the hydroxyl radical to seconds for peroxyl radicals. Collectively, ROS/RNS can lead to oxidation of proteins and DNA, peroxidation of lipids and ultimately cell death (Butterfield *et al*., 2001). Oxidation of proteins can induce changes in secondary and tertiary structure, protein aggregation and loss of function, susceptibility to proteolysis and fragmentation. Lipid peroxidation produces large amounts of aldehydes, such as 4-Hydroxynonenal, malondialdehyde, and acrolein, and leads to isoprostane formation (Butterfield *et al*., 2002).

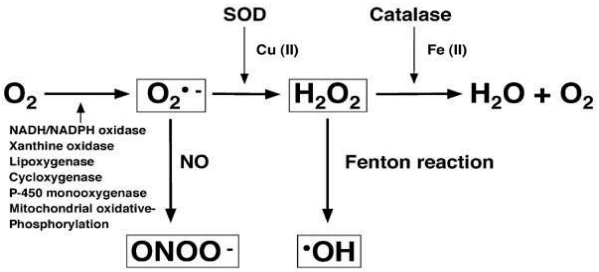

Fig.1: Reactive Oxygen Species formation. Sources of ROS generated endogenously by key metabolic pathways for these species. Multiple enzymes may induce ROS generation: NADH/NADPH oxidase, xanthine oxidase, lipoxygenase, cyclooxygenase, P-450 monooxygenase, and the enzymes of mitochondrial oxidative phosphorylation ($O_2^{-}\cdot$, superoxide anion radical; H_2O_2, hydrogen peroxide; ·OH, hydroxyl radical; ONOO-, peroxynitrite; SOD, superoxide dismutase). *J Med Invest (2001)*.

The production of ROS is associated with many forms of apoptosis (Suzuki *et al*., 1997), as well as with cell death occurring in stroke, ischemia, and many neurodegenerative diseases (Tan *et al*., 1998). Within

the central nervous system, glutamate toxicity is a major contributor to pathological cell death and appears to be mediated by ROS.

Among causes of ROS production it should be reminded the excessive activation of the glutamate receptors N-methyl-D-aspartate (NMDA), hence the abnormal Ca^{2+} influx through the receptor-associated cation channel that largely contribute to glutamate-mediated neuronal death (Lynch *et al*., 2002). The increase in intracellular calcium triggers robust activation of calcium-dependent-enzymes, including nitric oxide (NO) synthases (NOS), subsequent mitochondrial calcium overload and mitochondrial dysfunction, finally leading to ROS and peroxynitrite generation which initiate neuronal cell demise (Moncada *et al*., 2006; Corasaniti *et al*., 1992; Dawson *et al*., 1991; Dugan *et al*., 1995) (fig.2).

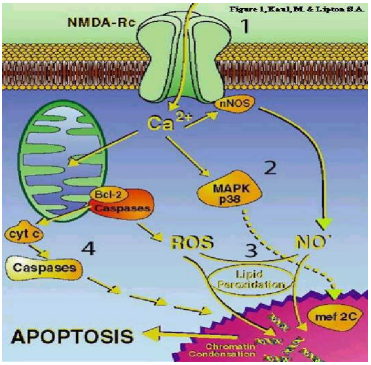

Fig.2: Current model of NMDA receptor-associated neuronal injury. Schematic illustration of the NMDAR-related signaling pathways that lead to neuronal apoptosis and may contribute to neurodegenerative disease. *NeuroAids(2000).*

In particular, the influx of Ca^{2+} through the NMDA receptor-gated cation channel activates the neutral cysteine protease calpain I that has been reported to have a role in excitotoxic neuronal death both *in vitro* and *in vivo* (Corasaniti *et al*., 1992).

Oxidative glutamate toxicity has been observed in primary neuronal cell cultures and neuronal cell lines. High concentrations of extracellular glutamate prevent cystine uptake into the cells, leading to depletion of intracellular cysteine and loss of glutathione (GSH) (Murphy *et al*., 1989).

The tripeptide glutathione (g-glutamate–cysteine–glycine, GSH), present in millimolar concentration in the brain, functions as antioxidant acting as ROS scavenger thus maintaining sulfhydryl groups of proteins in the

reduced form. Glutathione protects neurons against reactive oxygen species directly and indirectly, and binds to lipid peroxidation products, thereby providing neuroprotection (Pocernich *et al*., 2001).

A decreased GSH supply, corresponds to ROS accumulation and ultimately to chronic oxidative stress and cell death. Oxidative stress and the production of ROS are involved in the pathogenesis of a wide variety of chronic neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson disease (PD) and HIV-associated dementia (HAD) (Chava *et al*., 2005, de Vries *et al*., 2008). Alterations in tissue redox balance, associated with microglial activation, and abnormal protein deposition, are common pathological features of these diseases (Soto *et al*., 2003; Halliwell *et al*., 2006; Block *et al*., 2007; Lin *et al*., 2006; Lansbury *et al*., 2006). The deposition of amyloid-β, synuclein, huntingtin, and superoxide dismutase (sometimes connected with other proteins) has been shown in the brain of patients suffering from AD, PD, Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), respectively. The evidence for a role of oxidative stress in protein aggregates deposition is provided by the immunoreactivity of the pathological deposits with antibodies recognizing modified protein side-chains. Certainly free radical oxygen chemistry plays a pathogenetic role in all these neurodegenerative conditions, though it is as yet undetermined what types of oxidative damage occur early in pathogenesis, and what types are secondary manifestations of dying neurons (Sayre *et al.*, 2001).

Since oxidative stress has been implicated in the pathogenesis of several neurodegenerative disorders, therapies based on exogenous antioxidants administration have been developed. In this respect, it has been reported that high amounts of antioxidants are needed to achieve protective effects in the central nervous system (CNS), as most exogenous antioxidants do not efficiently cross the blood–brain barrier owing to their hydrophilic nature. Furthermore, administration of antioxidants is limited owing to their toxicity at high doses, resulting in a small therapeutic window of these agents (Moosmann *et al*., 2002). This emphasizes the need for alternative strategies to therapeutically counteract the detrimental effects of ROS and restore the cellular redox balance. A promising candidate to limit ROS-mediated damage is the activation of endogenous antioxidant enzymes present in the CNS (Agrawal *et al*., 2011), such as heme-oxigenase, catalase, glutamate cystein ligase, superoxide dismutase, and peroxiredoxins (Van Muiswinkel *et al*., 2005; Schreibelt *et al*., 2007).

1.2. Involvement of oxidative stress in HIV-Associated Dementia (HAD)

HIV-1 infection of the central nervous system (CNS) can lead to a variety of neurological problems, including motor disturbances, cognitive impairments and behavioural changes (McArthur *et al.*, 2004). These neurological symptoms can occur in various degrees of severity, and in their severest forms are termed HIV-Associated Dementia (HAD) (Navia *et al.*, 1986). It is estimated that one-third of adults infected with human immunodeficiency virus (HIV-1) develop dementia (Janssen *et al.*,1992). HAD is now the leading cause of dementia in people younger than 60 years of age (McArthur *et al.*,1993). HIV-associated cognitive impairment correlates with the increased presence in the CNS of activated, though not necessarily HIV-1 infected, microglia and CNS macrophages. This suggests that indirect mechanisms of neuronal injury and loss/death occur in HIV/AIDS as a basis for dementia since neurons are not themselves productively infected by HIV-1 (Ghafouri *et al.*, 2006). Many factors can contribute to the neuropathology of AIDS, particularly opportunistic brain infections (Almeida *et al.*, 2005). In the absence of opportunistic infections, major clinical symptoms include impaired short term-memory coupled with reduced ability of mental concentration, leg weakness, slowness of hand movement and gait as well as depression (Janssen *et al.*, 1992; Rackstraw, 2011). The HIV-1 associated neuropathology is microscopically characterized by the infiltration of macrophages into the CNS; the formation of microglial nodules; the presence of multinucleated giant cells which result possibly from virus-induced fusion of microglia and/or macrophages in central white and deep gray matter; reactive astrogliosis (Huang *et al.*, 2011); neuronal loss particularly in hippocampus, basal ganglia and caudate nucleus. In addition, a variable degree of white matter pathology with evidence of broad range of myelin and axonal damage , and the presence of HIV-1 in the cerebral spinal fluid (CSF) has been reported. (Lawrence *et al.*, 2002; Gonzalez *et al.*, 2005; Gendelman *et al.*, 1994). The human immunodeficiency virus-1 (HIV-1) is a member of the family of *Retroviridae* and carries a single-stranded RNA genome which is converted into DNA within the host cell. HIV-1 belongs to the primate lentivirus group in the *Lentivirus* genus. Several genomic clades (i.e. subtypes) of HIV-1 are recognized (A–G, K, 0) with different geographical distributions (Wainberg, 2004). Like all lentiviruses, HIV- 1 invades the central nervous system (Power et al., 2004), penetrating very soon after initial systemic infection, presumably persisting in the CNS for decades. According to the

"Trojan Horse hypothesis" (fig.5), HIV-1 enters the CNS, by migration of infected monocytes or of infected CD4+ T cells. Once the virus is in the brain, it can infect productively macrophages and microglia, whereas the infection of astrocytes is known to be restricted. The infection of neurons is yet questionable. As the prevalence of HIV-associated neurocognitive disorders (HAND) increases (Sacktor *et al*., 2002; McArthur *et al*., 2004; Joseph *et al*., 2005), studies of the mechanisms mediating the pathogenesis of Neuro-AIDS become even more important (Nath *et al*., 1998; Albright *et al*., 2003).

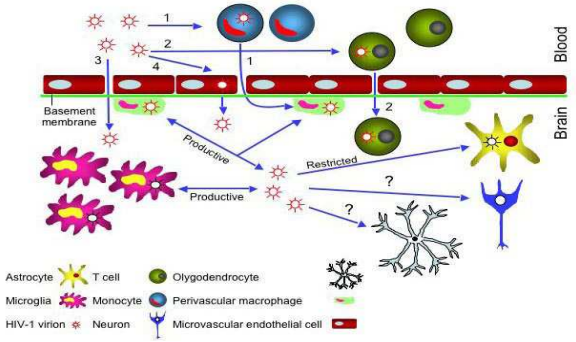

Fig.5: HIV-1 neuroinvasion. 1) According to the "Trojan Horse hypothesis" entry of HIV-1 into the brain takes place by the migration of infected monocytes which differentiate into perivascular macrophage. 2) The passage of infected CD4+ T cells can be another source of infection in the brain. Other probable causes of CNS infection might be: 3) the direct entrance of the virus or 4) entrance of HIV-1 by transcytosis of brain microvascular endothelial cells. Once the virus is in the brain it infects productively macrophages and microglia. Astrocyte infection is known to be restricted. The infection of oligodendrocytes and specially neurons is questionable. *Retrovirology (2006).*

The absence of significant neuronal infection by HIV-1 contrasts with the extensive neuropathological damage observed in HAD, therefore different mechanisms involving the HIV-1 infection of perivascular macrophages, microglia and possibly astrocytes might play the principal role in neuronal injury and the disruption of normal neurological function. The damage of neurons can result from their direct interaction with viral proteins, such as gp120, Tat (Transcriptional transactivator) and Vpr (viral protein R) produced by infected cells (Table 1.), or from an indirect effect due to the inflammatory process involving activated/infected monocytes, macrophages and astrocytes.

	Neurons	Astrocytes	Macrophages/microglia	BMVECs[c]
gp120	Activation of chemokine receptors; apoptosis	Global changes in gene expression; diminished glutamate uptake	TNF-α and IL-1 ⇑[b]; arachidonic acid metabolites ⇑; β-chemokines ⇑	Apoptosis
Tat	Depolarization of neurons; intracellular $[Ca^{2+}]$⇑; preventiation of long-term potentiation in the hippocampus; apoptosis	Improvement of cell survival; MCP-1 ⇑; iNOS⇑; VCAM-1 ⇑; ICAM-1⇑	TNF-α ⇑; chemoattraction of monocytes	Apoptosis; MCP-1⇑
Nef	Cell death; modulation of $[K^+]$ channel activities	Complement factor C3⇑;	Increased synthesis of various pro-inflammatory factors	Disruption of BBB[d]
Vpr	Apoptosis	Not found	Induction of RANTES/CCL5	Apoptosis

[a] For further details and references see text.
[b] ⇑: upregulation.
[c] BMVEC: brain microvascular endothelial cell.
[d] BBB: blood–brain barrier cell.

Table 1. Selected effects of HIV-1 proteins on brain cells which may contribute to CNS injury. *Virus Research (2005).*

The currently prevailing concept for damage of neurons during HIV-1 infection evokes the complex interplay of numerous soluble factors released by activated and infected cells. In particular, infected and/or activated glial cells release numerous factors of viral or cellular origin that directly or indirectly contribute to the injury into the CNS. Among these, chemoattractants promote infiltration of infected and/or activated monocytes and T-lymphocytes, then, other factors such as cytokines amplify the activation of glial cells that in turn release neurotoxic factors leading to neuronal injury and death. Neuronal death can be triggered by both excitotoxic and apoptosis-inducing factors. Excitotoxic factors cause overactivation of NMDAR-coupled channels, leading to excessive influx of Ca_2^+. The excess of glutamate is an excitotoxic condition due to the release of glutamate from injured neurons and to the impaired glutamate uptake of infected astrocytes (Fig.6).

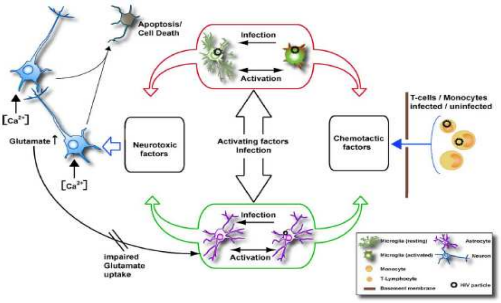

Fig. 6. Model for HIV-1 related injury to the central nervous system. Infected and/or activated glial cells release numerous factors of viral or cellular origin that directly or indirectly contribute to injury in the CNS. Among these, chemoattractants promote infiltration of infected and/or activated monocytes and T-lymphocytes, activating factors amplify activation of glial cells, and neurotoxic factors lead to neuronal injury and death. Neuronal injury and death are caused by excitotoxic and apoptosis-inducing factors. Excitotoxic factors cause overactivation

of NMDAR-coupled channels, leading to excessive influx of Ca_2^+ and neuronal injury. Excess glutamate is excitotoxic and caused by release of glutamate from injured neurons and by impaired glutamate uptake of infected astrocytes. This model is highly simplified. *Virus Research (2005).*

Oxidative stress is thought to play a role in the onset of HIV-associated dementia as suggested by increased protein and lipid peroxidation in the brain and cerebrospinal fluid of HAD patients compared to seropositive non-demented patients. Moreover nitrated tyrosine residues, evidence of peroxinitrite reaction with proteins, is increased in HAD brains (Boven *et al*.,1999), indicating the presence of chronic oxidative stress.
It has been shown that the concentrations of GSH and other sulfhydryl compounds are decreased in the blood, liver, and central nervous system (CNS) of HIV-infected patients (Castagna *et al*., 1995; Choi *et al*., 2000), and low GSH is associated with poor survival of HIV-infected patients, whereas the administration of GSH to the patients decreases mortality (Herzenberg *et al*., 1997). The ROS scavenger N-Acetyl-l-cysteine (NAC) acts as an indirect precursor of glutathione by raising intracellular levels of cysteine, a precursor of glutathione (Pocernich *et al*., 2000; Pocernich *et al*., 2001), furthermore, NAC also has antioxidant properties of its own due to the sulfhydryl group. Interestingly, the administration of NAC to HIV-infected patients has been shown to decrease mortality (Herzenberg *et al*., 1997). Recently, various antioxidants as NAC analogs, including N-(N-acetyl-l-cysteinyl)-S-acetylcysteamine, or selegiline drug (Merino *et al*., 2011) have been shown to increase glutathione and display anti-HIV activity making them possible therapeutic candidates for HIV infection (Drake *et al.*, 2002; Drake *et al.*, 2003). Different factors of both viral and cellular origin have been reported to induce directly or indirectly oxidative stress in different type of cells, such as astrocytes, neurons and brain endothelial cells (cytokines, chemokines, virotoxin: gp120, Tat, Nef and Vpr). Among these, the viral protein Tat is a neurotoxin that after being secreted from HIV-infected cells, can interact with receptors of cells and/or taken up by infected and uninfected cells thus interfering with normal cellular functions (Liu et al., 2000; Ensoli et al.,1993; Sabatier et al., 1991).

1.3. Neurotoxicity induced by HIV-1 Tat

HIV-1 is the etiologic agent of acquired immunodeficiency syndrome (AIDS) (fig.7).

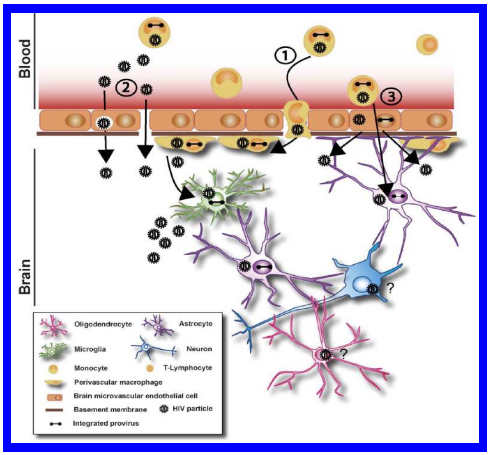

Fig.7: Models for invasion of the brain by HIV-1 at the blood–brain barrier. Three pathways have been proposed for HIV-1 entry into the brain at the blood–brain barrier. The most favoured hypothesis is (1) migration of infected cells, primarily monocytes, from the blood into the brain ("Trojan horse" hypothesis). Other pathways proposed for entry of HIV-1 are (2) passage of free virus into the brain either by migrating between or transcytosis through brain microvascular endothelial cells (BMVECs); (3) release of virus into the brain by infected BMVECs. Virus released by infected perivascular macrophages contributes to productive infection of microglia and promotes spread of virus in the parenchyma of the brain. Restricted infection of astrocytes can occur at the blood–brain barrier or in the brain parenchyma by contact with free virus or with virus-infected cells. Infection of neurons and oligodendrocytes is controversially discussed (indicated in figure by question mark (?).*Virus Research* 111: 194–213.

HIV-1 gene expression and transcription are crucial steps in the viral replication cycle, which is considered to be a potential target for inhibition of HIV-1 (Nekhai *et al*., 2006; Richter *et al.*, 2006). Replication of HIV-1 is controlled by a variety of viral and host proteins. The transcriptional regulation of the virus is a complex event that requires the cooperative action of both viral (e.g., Tat) and cellular (e.g., C/EBPβ, NF-κB) factors. The HIV-1 viral genome encodes for three structural genes (gag, pol, and env), three regulatory genes (tat, nef, and rev), and three accessory genes (vpr, vpu, and vif), all in overlapping frames. These genes are flanked by 5′

and 3′ long terminal repeats (LTRs) that contain enhancer and promoter elements essential for proviral transcription and replication (Karn *et al*., 1999) (Fig.8).
The HIV-1 Trans-Activator of Transcription (Tat) is an RNA-binding protein that contributes to transactivation of viral and cellular genes (Ju *et al*., 2009; Mahlknecht *et al*., 2008; Nekhai *et al*., 2007). Tat is an early regulatory protein that facilitates HIV-1 transcription and replication and has a variable length of 86–104 aa, encoded by two exons (fig.8).

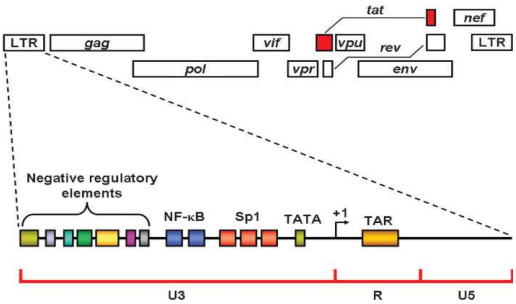

Fig. 8: Structure of the HIV-1 genome. Tat is encoded by two exons (in red). The first exon codes for the first 72 aa, which are sufficient for Tat transactivation. The second exon codes for amino acids 73–104. The detailed structure of the LTR is shown at the bottom of the picture. The HIV-1 LTR contains many protein-binding sites. Some of the protein-binding sites, such as negative regulatory elements, downregulate transcription of the HIV-1 genome through binding to regulatory proteins. The HIV-l LTR also contains a TATA box, two direct repeats of NF-kB-binding site and three tandem repeats of Sp1-binding site. Tat recruits transcription factors on the LTR to upregulate the transcription of the HIV-1 genome. Journal of General Virology (2010), 91, 1–12

The first exon encodes the first 72 aa. Due to the variable length of Tat, its molecular weight varies from 14 to 16 kDa (Pugliese *et al*., 2005).
Incomplete forms of this viral protein (from 58 to 72 aa) may also be able to induce the biological effects of the full length protein. A double splicing mechanism occurs after the transcription of tat mRNA. This is a post – transcriptional modification that consists of cutting of the tat mRNA and removal of unnecessary sequences. The process is followed by the joining together of nucleic acid sequences (Pugliese *et al*., 2005).
The extracellular form of Tat, which is released from productively infected cells, is also able to enter target cells and induce its effects (Ferrari *et al*., 2003; Zheng *et al*., 2005). Mutational analysis of HIV-1 Tat has identified two important functional domains: an activation domain that mediates its interactions with cellular machinery and an arginine-rich region that is

required for binding to the transactivation responsive element (TAR) RNA (Hwang *et al*., 2003). Tat acts in concert with host cellular factors, such as the human positive transcription elongation factor P-TEFb, consisting of cdk9 and cyclin T1, to stimulate transcriptional elongation from the viral LTR through a specific interaction with the TAR element, a 59-residue stem–loop RNA (Karn *et al*., 1999).

Oxidative stress is thought to play a role in the onset of HIV-dementia and it has been recently demonstrated to be associated with Tat-induced neurotoxicity **(**Chava *et al*., 2005; Butler *et al.,* 2011**)**. Tat was found to induce oxidative stress directly and indirectly (Bizhan *et al*., 2010). Tat is actively secreted into the extracellular environment mainly from astrocytes, microglia, and macrophages, and is taken up by neighboring uninfected cells such as neurons (Chang *et al*., 1997; Ensoli *et al*., 1993; McArthur *et al.,* 1993). The HIV-1 protein Tat released from astrocytes reportedly produces trimming of neurites, mitochondrial dysfunction, and cell death in neurons (Chauhan *et al*., 2003).

A single injection of full-length Tat (1–86), Tat (1–72), or the short basic domain of Tat (48– 57) into the hippocampus or thalamus resulted in glial cell activation, influx of inflammatory cells, induction of inducible nitric oxide synthase and neurotoxicity (Jones *et al*., 1998; Philippon *et al*., 1994). Tat toxicity, assessed by *in vitro* systems between 100 and 500 nM concentration, is thought to be mediated through excitotoxic mechanisms involving calcium influx, mitochondrial calcium uptake, generation of ROS, activation of caspases and eventually apoptosis (Kruman *et al*., 1999). Oxidative stress has been demonstrated both in the brain and in cerebrospinal fluid (CSF) of HAD patients (Turchan *et al*., 2003). Brain tissues are particularly susceptible to Tat toxicity (Pocernich *et al*., 2005). Evidently, Tat plays an important role during neuropathogenesis, both as an intracellular and extracellular mediator of neurotoxicity (Wong *et al*., 2005). Price *et al*. (2005) have demonstrated that Tat causes oxidative stress in immortalized rat brain endothelial cells by a time-dependent decrease in the levels of intracellular glutathione and a time-dependent increase in the levels of the oxidized form of glutathione (GSSG). As a result of the oxidative stress induced by Tat, glutathione is then oxidized to GSSG.

HIV-1 Tat-mediated neurotoxicity is directly evident from a study in which was demonstrated that cell death was completely prevented when the supernatant from HIV-infected monocytes was first immune absorbed with antiserum to Tat and the HIV-1 protein gp120 (Turchan *et al*., 2001). The findings set out above in paragraphs suggest that Tat is capable of directly exciting neurons and causing excitotoxicity. However, Tat toxicity is also

related to glutamate receptor activation, since antagonists of NMDA and non-NMDA receptors partially protect neurons from the toxic effects of Tat (Haughey *et al*., 2001; Hayman *et al*., 1993; Magnuson *et al*., 1995). Neurotoxic effects of Tat are in part mediated by direct interactions with a polyamine-sensitive site on the NMDA receptor (Prendergast *et al*., 2002; Self *et al*., 2004). Co-localization analysis by Chandra *et al*. (2005) demonstrates large immunoreactive patches of Tat in close proximity to and sometimes partially overlapping with patches of NMDA receptor immunoreactivity, demonstrating a partial co-localization of the Tat protein with the NMDAR (Chandra *et al*., 2005). Neurodegeneration in HAD occurs in uninfected neurons at sites that are often distant from the site of viral replication (Pocernich *et al*., 2005). In particular, Tat could directly interact with neurons, after being released into extracellular space by infected glia/macrophages within the brain (Chang *et al*., 1997). Tat may also activate astrocytes to induce the expression of inducible nitric oxide synthase (iNOS) (Liu *et al*., 2002), leading to the formation of an excess of nitric oxide. Tat-activated glial cells and HIV-infected macrophages are able to secrete several cytokines such as TNF-α, which also contributes to iNOS induction thus leading to increased NO production (Bukrinsky *et al*., 1995). NO excess also enhances glutamate release from astrocytes (Bal-Price *et al*., 2002), exacerbating excitotoxicity. Overproduction of NO is proposed, as reported in many studies, to increase HIV-1 replication, on the contrary low levels of NO can inhibit HIV-1 replication (Persichini *et al*.,1999; Torre *et al*., 2002).

1.4. Metabolism of polyamines and oxidative stress in the brain

Polyamines are ubiquitous small, positively charged, basic molecules (Fig. 9), the only polyamines synthesized in mammalian cells are putrescine, spermidine and spermine.

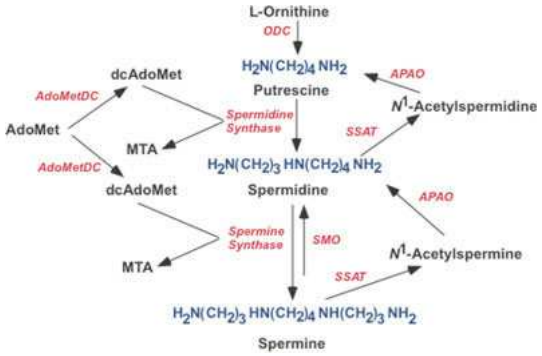

Fig.9: Polyamine structures, biosynthesis and interconversion. Polyamine structures are shown in blue. Enzymes are shown in red italic fonts (ODC, L-ornithine decarboxylase; AdoMetDC, Sadenosylmethionine decarboxylase; SMO, spermine oxidase; SSAT, spermidine/spermine-N1-acetyltransferase; APAO, acetylpolyamine oxidase). IUBMB Life 2009.

Polyamine levels are controlled by changes in the content of the key enzymes shown in Fig. 9. The induction of SMO that occurs in response to polyamine analogues (Wang *et al*., 2006), TNF-α (Babbar *et al*., 2006) and during cell differentiation leads to decreased spermine content (Cervelli *et al*., 2009). Flux through APAO activity is normally regulated by the availability of the substrates provided by SSAT rather than by changes in APAO activity. Alterations in efflux and uptake mechanisms also play an important role in maintaining cellular polyamine content. There are transport systems for both the uptake of polyamines and for their efflux, but are currently poorly understood at the biochemical level. Polyamine transport can follow a dynamin-dependent and clathrin-independent endocytic uptake pathway.

Both APAO and SMO are flavoproteins that generate reactive aldehydes and H_2O_2 and may cause oxidative damage (Casero *et al*., 2010). This may be a more serious problem with SMO since APAO is located in peroxisomes (Pegg *et al*., 2009). In neurodegenerative diseases

augmented polyamine metabolism results in the generation of hydrogen peroxide and a number of reactive aldehydes that participate in the death of compromised tissue. The concept of "oxidative stress" has become a mainstay in the field of neurodegeneration but has failed to differentiate critical events from epiphenomena and sequelae.
Polyamines play important roles in the regulation of ion channels (Fig.10).

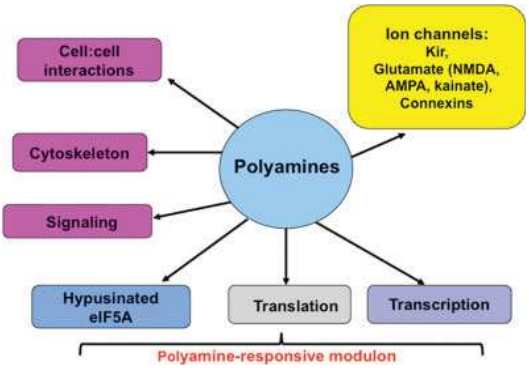

Fig.10: Functions of polyamines. Polyamine levels affect ion channels, cell-cell interactions, the cytoskeleton, signalling via phosphorylation and other mechanisms, activity of eIF5A via the role of spermidine as a precursor for its hypusination, transcription and mRNA translation. The effects on transcription and translation (both direct and indirect) alter the cellular levels of many proteins making up the polyamine-responsive modulo as described by Igarashi and coworkers. IUBMB Life 2009.

Both glutamate receptor ion channels, which mediate excitatory synaptic transmission in the mammalian brain, and inwardly rectifying potassium channels (Kir), which control membrane potential and potassium homeostasis in many cell types, are affected as well as certain connexin-linked gap junctions and some other channels that affect intracellular calcium signalling (Lynch *et al*., 2002; Moncada *et al*., 2006; Corasaniti *et al*., 1992; Dawson *et al*.,1991). Polyamines influence glutamate receptors mediating slow voltage-dependent responses such as NMDA receptors, and those producing fast responses at excitatory synapses, such as a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainate receptors (Lynch *et al*., 2002).
The NMDA receptors are involved in synaptic plasticity and may also play a role in seizure activity. The endogenous polyamines spermine and spermidine have been shown to have both stimulatory and inhibitory effects on NMDA receptor (Williams *et al*., 1989). Although both polyamines are

found in high concentration in the brain, their functions remain still largely unknown (Williams *et al*., 1994). The extent of the effects either stimulatory or inhibitory of spermine on NMDA receptors of cultured neurons is highly variable and likely due to the expression of different subunits of the NMDA receptor complexes. Williams *et al*. (1994) provided evidences that high molecular concentration of spermine would increase the activation of some subtypes of NMDA receptor. Thus, polyamines may play a role in synaptic transmission involving NMDAR.

Spermine (and, to a lesser extent, spermidine) has multiple effects on these receptors including stimulation and a weak voltage-dependent inhibition representing an open-channel block. Spermine stimulation, which occurs via binding to the extracellular R domain, causes an enhancement of the current gated by glutamate and glycine. There is a 'glycine-dependent stimulation', which produces an increase in the affinity of the receptor for glycine. At physiologic pH, spermine stimulatory effects are seen at NR1/NR2B receptors but not at NR1/NR2A receptors (Dugan *et al*., 1995). The spermine block occurs via interactions in the outer vestibule of the channel pore (Tan *et al*., 1998). The AMPA-type glutamate receptors are responsible for fast excitatory neurotransmission in the CNS. Thus, polyamines may regulate the amount of Ca_2 flux and the excitability threshold at developing synapses. Similar to its action on NMDA receptors, spermine potentiates kainate receptors by relieving proton inhibition of the receptor (Calkins *et al*., 2009).

On the other hand, the polyamine-sensitive subtype of the NMDA receptor seems to be specifically involved in Tat-elicited excitotoxicity. In particular, it has been reported that Tat can act as an agonist of the NMDA receptor in a clade-dependent manner by directly interacting with the receptor, thus leading to persistent NMDAR activation. To note, this effect was only observed with clade B-Tat. Interestingly, patients infected with HIV-1 clade C do not develop severe forms of dementia, unlike HIV-1 clade B-infected people which are more frequently affected by severe neurocognitive impairment.

1.5. The antioxidant cell response: KEAP-1/Nrf2/ARE Signaling Pathway

NF-E2-related factor-2 (Nrf2), a member of the cap 'n' collar family of basic leucine zipper transcription factors, forms heterodimers with the small Maf proteins and binds to the antioxidant response element (ARE) (fig.3).

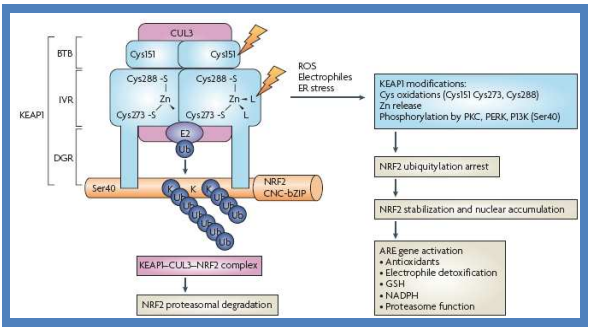

Fig.3: KEAP1/NRF2interaction. The dimeric Kelch-like ECH-associated protein-1 (KEAP1) receptor contacts one NRF2 (nuclear factor (erythroid-derived-2)-like-2) molecule at two distinct N-terminal sites[103, 131]. The KEAP1–cullin-3 (CUL3) complex constantly targets NRF2 for proteosomal degradation through ubiquitylation of NRF2 Lys residues that are located at its N terminus, between the two KEAP1 interaction sites. Electrophiles and signals from reactive oxygen species (ROS) modify KEAP1 Cys residues, leading to zinc release and a conformation that is non-permissible for ubiquitylation. An alternative speculative model proposes that two NRF2 molecules are each contacted by the Kelch domain of dimeric KEAP1 (Ref. 104). Phosphorylation of Ser40 by phosphatidylinositol 3-kinase (PI3K), protein kinase RNA (PKR)-like endoplasmic reticulum (ER) kinase (PERK) or protein kinase C (PKC) might also lead to ubiquitylation arrest. KEAP1 dimerizes through the broad complex–tramtrack–bric-a-brac (BTB) domain and interacts with NRF2 through the Kelch domain. The KEAP1 intervening region (IVR) that carries reactive Cys residues is located between the BTB and Kelch domains. ARE, antioxidant response element; CNC-bZIP, cap 'n' collar-basic leucine zipper; DGR, double glycine repeat; GSH, glutathione. Nature Reviews. Molecular Cell Biology 2007.

The antioxidant response element (ARE) are enhancer located in the 5′ flanking region of many phase II detoxification genes and was first identified by T.H. Rushmore and colleagues (Rushmore *et al*., 1990; Rushmore *et al*., 1991). Nrf2 plays a critical role in the constitutive and inducible expression of numerous detoxifying and antioxidant genes, including NAD(P)H:quinone oxidoreductase (NQO1), glutamate-cysteine ligase (GCL, also known as glutamyl-cysteine synthetase) and heme oxygenase-1 (HO-1), through activation of the ARE in the regulatory region

of the genes (Venugopal *et al*., 1996; Itoh *et al*., 1997; Yang *et al*., 2005). Evidence has also shown that the basal activity of Nrf2 is suppressed by its cytoplasmic repressor Kelch-like ECH-associated protein1 (Keap1) (Itoh *et al*., 1999). Advances in this field suggest that Keap1 has a role in both Nrf2 cytoplasmic sequestration and its ubiquitin-mediated proteolysis (Kobayashi *et al*., 2004; Cullinan *et al*., 2004). Small molecules induce Nrf2- dependent ARE activation inducing the release of Nrf2 from Keap1 and the translocation of Nrf2 to the nucleus. Once in the nucleus Nrf2 binds to small Maf proteins in order to elicit the transcription of the ARE genes (Itoh *et al.*, 1997; Motohashi *et al.*, 1997; Chan *et al*.,1999). Activation of this pathway has been studied in a variety of tissues and cell types using many chemical activators (fig.4).

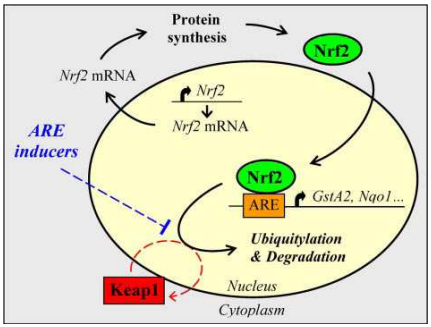

Fig.4: Proposed Nrf2-ARE signaling pathway. Nrf2 is expressed constitutively in the cell and translocates directly to the nucleus following its synthesis. Following transactivation of its genes, Nrf2 is targeted for degradation by Keap1 in the nucleus, a process that requires the transient shuttling of Keap1 into this compartment. In cells under stress, stabilization of Nrf2 is thought to be dependent on mechanisms that either prevent or reduce access of Keap1 to Nrf2. J Biol Chem. 2009.

Induction of Nrf2-mediated transcription, particularly in astrocytes, has been shown to protect against neurotoxicity from a variety of insults. In primary neural cultures, Nrf2 activation is known to be neuroprotective against oxidative stressors and mitochondrial toxins such as hydrogen peroxide and tert-butyl hydroperoxide (Calkins *et al*., 2009). In all cases, either Nrf2 activation was shown to contribute to neuroprotection, or conversely, Nrf2 deficiency led to increased sensitivity. These observations illustrate the critical concept that Nrf2 response is an inducible and highly protective mechanism that may be employed by cells in culture or *in vivo* to combat oxidative stress (Johnson *et al*., 2002; Agrawal *et al*., 2011).

Various reports have underscored the cytoprotective effects of Nrf2-induced antioxidant protection in distinct CNS cell types and animal models for neurodegeneration. It has been reported that Nrf2-driven gene expression of antioxidant enzymes was able to protect primary astrocytes from hydrogen peroxide-induced apoptosis, conversely decreased levels resulted in augmented susceptibility to oxidative stress in the same cells (Lee *et al*., 2003). Interestingly, astrocytes overexpressing Nrf2 are able to rescue neurons from oxidative stress, indicating that astrocytes are the predominant cell type involved in Nrf2-mediated protection (Lucius *et al*., 1996; Desagher *et al*., 1996). The Nrf-2 ARE pathway is found in most tissues and controls the expression of a multitude of genes involved in protecting cells (Kensler *et al*., 2007). The role of Nrf2 as a potential therapeutic target for ROS-dependent neurodegenerative disorders has been recently recognized (Calkins *et al*., 2009). Interestingly, it was very recently shown that the activation of the Nrf2/ARE pathway protects against oxidative stress in HIV-1 transgenic rats (Fan *et al*., 2011).

2. AIM OF THE WORK

HIV Associated Dementia is a neurological syndrome that occurs in the late stage of the HIV-dependent infection and is characterized by abnormalities in cognition, motor performance and behavior. Glial cells (specifically microglia and astroglia) are the target and reservoirs for persistent infection (Takahashi *et al*., 1996), playing a principal role in the neuropathogenesis of HAD. Neurons are rarely infected although they are the final targets of disease. This has led to the concept that the pathogenesis of HAD is caused not by direct infection of neurons, but by secretory and cell-associated products (viral and cellular) manufactured by glia, that disrupt neuronal function (Turchan-Cholewo *et al.*, 2009).

Several investigators have studied how viral proteins such as gp120 and Tat may contribute to disease (Giulian *et al*., 1993; Bagetta *et al*., 1994; Lannuzel *et al*., 1997; Hesselgesser *et al*., 1998), focusing the attention on possible indirect mechanisms, including oxidative stress (Mollace *et al*., 2001 Rackstraw, 2011; Mattson *et al*., 2005; Butterfield *et al*., 2006; Steiner *et al*., 2006).

Literature data on the association between polyamine oxidase activity and oxidative stress-induced neurodegeneration (Amendola *et al.*, 2005; Seiler *et al.*, 2000) prompted us to investigate a possible involvement of polyamine metabolism in HIV-Tat-induced neuronal toxicity. In particular, we assume that a role can be devised for polyamine-derived ROS generation in Tat-induced oxidative stress. Thus, we wondered whether Tat might upregulate the expression and the activity of SMO and APAO enzymes in astrocytoma and neuroblastoma cell culture models and whether this effect could be related to Tat-induced cell death by using ROS scavengers and specific polyamine oxidases inhibitors. Increased ROS levels can promote cell damage especially in neurons that seems to be more sensitive to oxidative stress-induced cell death with respect to astrocytes. It is well established that astrocytes can activate an efficient Nrf2-dependent antioxidant cell response thus maintaining the redox balance and preserving them from cell death. Therefore we assumed a different ability of astrocytoma and neuroblastoma cells in activating the antioxidant cell response after Tat exposure, which in turn should lead to a selective death of neurons. In this context, aim of the second part of this research project was to study the activation of the Nrf2/ARE pathway in Tat-treated or-transfected cells.

3. RESULTS

Selective toxicity of HIV-Tat recombinant protein on SH-SY5Y and NIE-115 neuroblastoma cell lines

Many evidence indicates that HIV-1 Tat protein plays a key role in the neurotoxicity occurring in HIV-1 infection. In particular, Tat can directly interact with the polyamine sensitive subtype of the NMDAR (Prendergast *et al*., 2002), thus explaining the selective death of neurons.
Oxidative stress is also thought to be involved in the mechanism leading to neuronal loss observed in HAD patients, although, the origin of the unbalanced redox state is not yet clearly demonstrated.
Thus, we first examined the ability of HIV-1 Tat recombinant protein to induce cell death and ROS production in astrocytes and neuroblastoma cell culture models.
To this aim we used U373-MG astrocytoma as well as SH-SY5Y and NIE-115 neuroblastoma cells, the latter two expressing the NMDA subtype of glutamate receptors (Sun and Murali, 1998; Halliwell *et al.*, 1989).
We selected the neuroblastoma cell lines also because of their susceptibility to NMDA-induced cell death (Corasaniti *et al*., 2007; Norby *et al*., 1997).
The effect of Tat treatment on cell viability was evaluated by MTT assay (Bruggisser *et al*., 2002).
Neuroblastoma and astrocytoma cells were treated with recombinant Tat (100 ng/ml) for 24 and 48 h.
As shown in Fig. 1, a significant decrease (30%) of cell viability has been observed early as 24 h of treatment only in neuroblastoma cells (SH-SY5Y). Whereas U373 glioma cells were resistant to Tat-induced toxicity for all the time points investigated.
In order to analyze whether the reduced viability induced by Tat was mediated by ROS generation, we performed MTT assay on Tat-treated cells in the absence or presence of the ROS scavenger NAC.

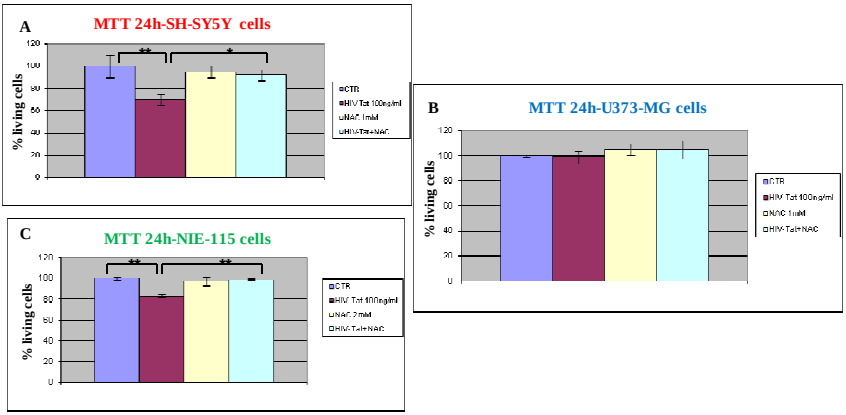

Fig.1: **MTT vitality assay**. Glioma (U373-MG) (**B**) and neuroblastoma (SH-SY5Y and NIE-115) (**A,C**) cells were treated with 100 ng/ml HIV-1- Tat recombinant protein. MTT assay was performed 24 h after treatments. 2 mM NAC pre-treatment demonstrated ROS toxicity-dependent cell death. Data were expressed as means ± S.D of three different experiments, each with two replicates; **p*<0.01, ***p*<0.001.

As reported in Fig. 1, the pretreatment with NAC completely restored cell viability in both the neuroblastoma cell lines.

These results support that Tat affected cell viability through a burst of oxygen free radicals. Therefore, we investigated the levels of intracellular ROS using the same cell culture models.

To this aim we used CM-H2DCFDA, a cell-permeant indicator that detects mainly H_2O_2. We first evaluated intracellular ROS generation in cells treated with recombinant Tat (100 ng/ml) for 1 h.

As shown in Fig. 2, ROS levels were significantly increased mainly in SH-SY5Y neuroblastoma cells rather than in U373 glioma cells treated with Tat with respect to untreated cells.

ROS generation was completely prevented by 1-h pre-treatment with the ROS scavenger NAC (2 mM).

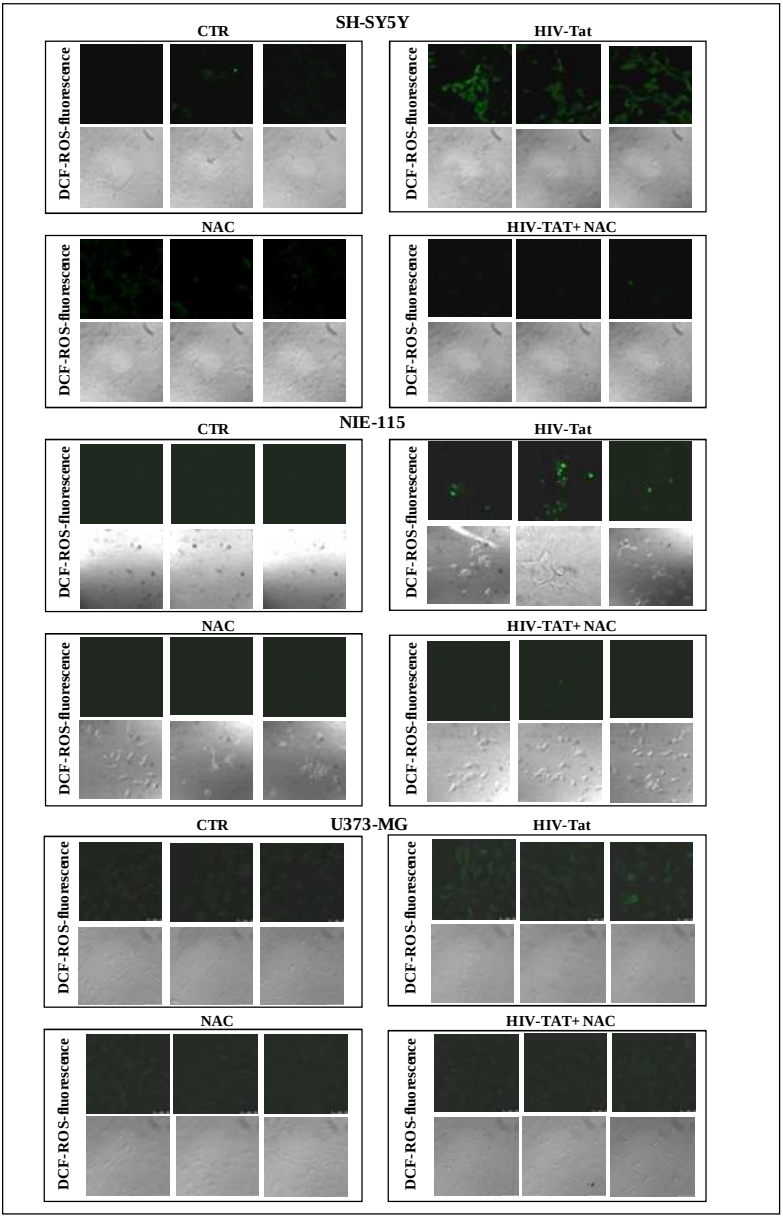
SH-SY5Y
CTR
HIV-Tat
NAC
HIV-TAT+ NAC
NIE-115
CTR
HIV-Tat
NAC
HIV-TAT+ NAC
U373-MG
CTR
HIV-Tat
NAC
HIV-TAT+ NAC
DCF-ROS-fluorescence

Fig.2: **characterization of HIV-1 Tat-induced cytotoxicity in neuroblastoma cultures: DCF-ROS determination.** HIV-1 Tat recombinant protein (100 ng/ml) induced accumulation of intracellular ROS more in neuroblastoma cells than in astrocytes, already 1 h after treatments. 2 mM NAC pre-incubation prevented, at least in part, ROS increase, demonstrating ROS-dependent fluorescence increase. Fluorescence images were acquired by an inverted DMI 6000 confocal scanner microscope TCS SP5 (Leica Microsystems CMS GmbH) with a 40 and 63X oil immersion objective. Images were acquired using Leica application suite advanced fluorescence software, images were acquired from three chosen field. Results were confirmed by three independent experiments.

Analysis of PA-oxidases gene expression and evaluation of spermine oxidase activity in neuroblastoma cells treated with HIV-Tat

Since the polyamine sensitive subtype of NMDA receptor has been implicated in Tat-induced neurotoxicity, we wondered whether the ROS production elicited by Tat could be the consequence of augmented polyamine metabolism. Thus we performed a study to deeply investigate the involvement of polyamine metabolism in neurotoxicity elicited by Tat. Firstly, we analyzed by RT-PCR the expression of PA enzymes in neuroblastoma cells treated with HIV-1 Tat (100 ng/ml).
The results shown in Fig. 3 indicate the up-regulation of APAO and SMO mRNA after an 8-h treatment with the recombinant viral protein, suggesting a putative role for polyamine oxidases in mediating Tat-induced neurotoxicity.

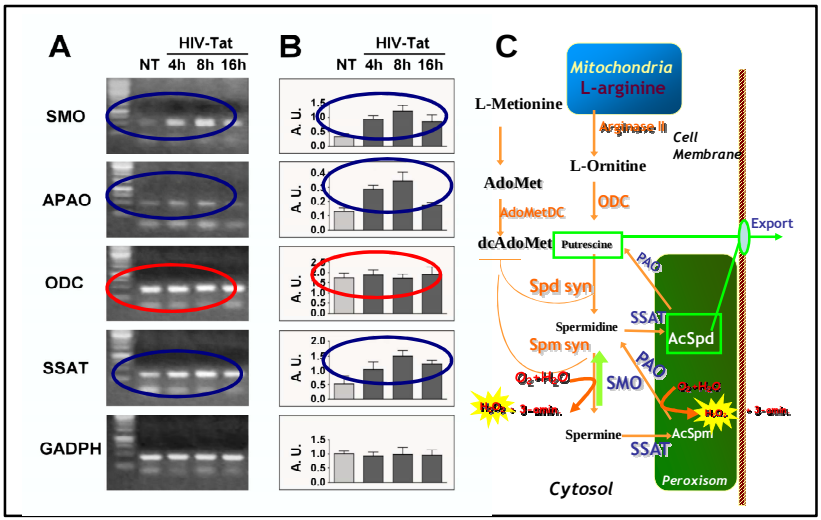

Fig.3: **(A and B) involvement of polyamine oxidases enzymes in HIV-1 Tat-induced toxicity.** HIV-1 Tat recombinant protein (100 ng/ml) induced up-regulation of SMO and APAO mRNA already 8 h after treatments in neuroblastoma cells. RT-PCR results were confirmed by three independent experiments and data expressed as means ± S.D. **C)** Polyamine metabolism scheme. Representation of polyamine metabolism and derived secondary toxic products.

Both APAO and SMO are flavoproteins that generate reactive aldehydes and H_2O_2 as byproducts and may cause oxidative damage (Wood *et al.*, 2007). In particular SMO, the most recently characterized enzyme involved in polyamine metabolism, catalyzes the direct back-conversion of spermine to spermidine in an FAD-dependent reaction that also yields the byproducts hydrogen peroxide and 3-aminopropanal. Since these metabolites have been implicated in neurodegeneration by in vitro and in vivo studies, we analyzed the activity of SMO on SH-SY5Y cells after 1 and 4-h treatment with Tat (100 ng/ml).
The results shown in Fig. 4 demonstrate a significant increase of SMO activity in Tat-stimulated cells and suggest a relationship between HIV-Tat neurotoxicity and polyamine metabolism.

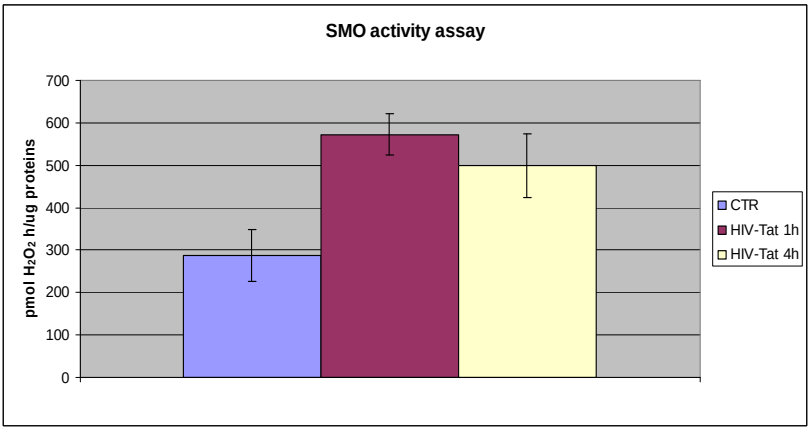

Fig.4: SMO activity assay. Neuroblastoma SH-SY5Y were treated with HIV-1 Tat (100 ng/ml) for 1 and 4 h. The SMO activity was measured spectrophotometrically by following the formation of a pink adduct as the result of the H_2O_2-dependent oxidation of 4-aminoantipyrine catalyzed by horseradish peroxidase. Data reported are the average of three different experiments, each with two replicates, standard deviation (SD) was 5%.

Involvement of SMO/APAO enzyme activity in Tat-induced ROS generation and cell death

In order to assess the involvement of polyamine metabolism-derived H_2O_2 in Tat-induced cell death, we performed experiments to inhibit SMO and APAO activity in cells treated with Tat. To this aim we chose the two polyamine analogues, MDL-72527 a well known spermine inhibitor (Seiler *et al.,* 2000) and chlorhexidine (CHX), a recently proposed spermine analogue whose inhibitory effects on SMO and APAO enzymes were supported by *in vitro* study and molecular modelling (fig. 1 of supplement). Firstly, we performed cell treatments with several doses of CHX, ranging from 0.1 to 1 mM, to ruled out a direct cytotoxic effect of the compound. At doses higher than 10 µM we observed a lethal effect, whereas at lower concentration (100 nM) CHX unaffected cell viability until 72 h post-treatment (fig. 5).

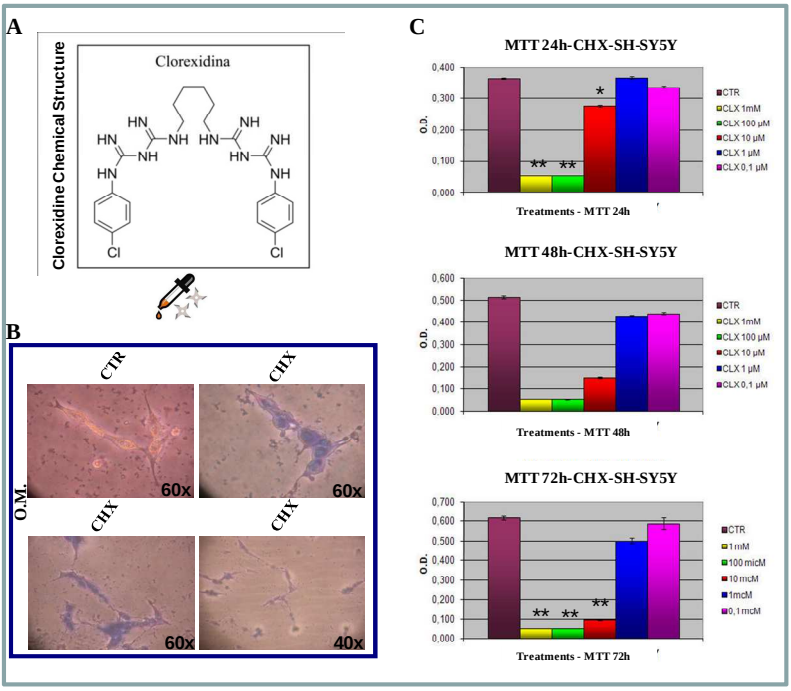

Fig.5: neuroblastoma cells and CHX. A) Chlorhexidine chemical structure; **B)** Trypan Blue exclusion test was performed on SH-SY5Y cells after 24-h treatment with 1 mM CHX. Death cells were selectively colored. **C)** MTT vitality assay was performed to test toxicity-dependent cell death by CHX. Neuroblastoma (SH-SY5Y) cells were treated with 0,1µM-1mM CHX and MTT assay was performed after 24, 48 and 72-h treatments. Data were expressed as O.D. means ± S.D of three different experiments, each with two replicates; CTR vs. treatment =**p*<0.01, ***p*<0.001 .

Thus, in order to analyze the effect of polyamine oxidases inhibition on HIV-1 Tat-induced ROS production and cell death, we used CHX at 100 nM concentration to pretreat overnight human neuroblastoma cell lines. As expected, Fig. 6 shows that both 50 µM MDL and 100 nM CHX pre-treatments prevented ROS increase (fig.6, fig. 2 of supplement) in SH-SY5Y and N1E-115 cells. The effect on cell viability has been evaluated only on CHX-pretreated cells.

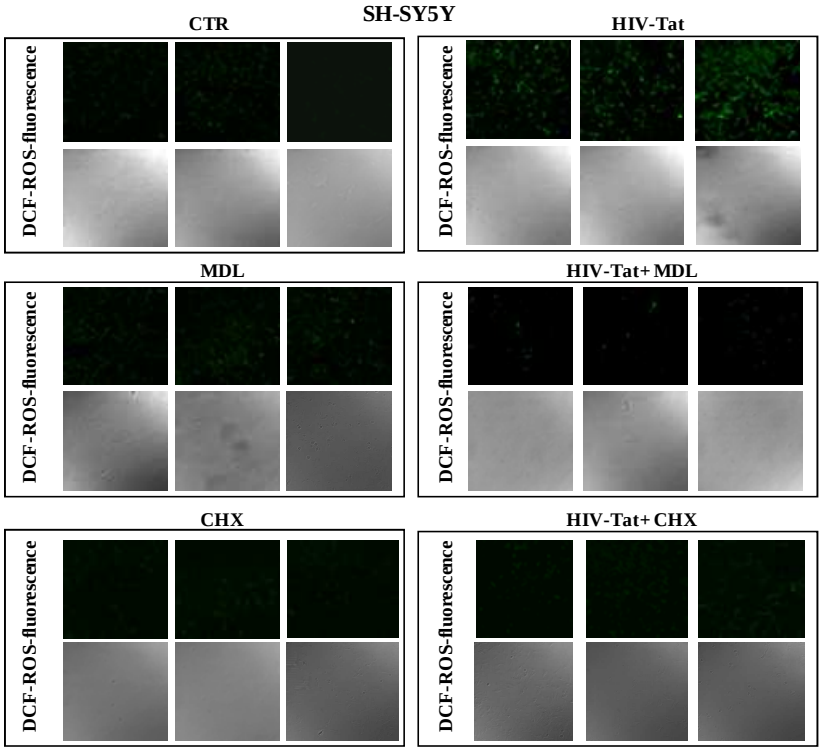

Fig.6: **polyamine oxidases inhibitors prevent HIV-1 Tat-induced ROS increase.** 100 ng/ml HIV-1 Tat-induced ROS intracellular accumulation was prevented by a 3-h pre-treatment of SH-SY5Y with 50 µM MDL and 100 nM CHX. ROS were measured using DCF-DA fluorescence probe. Fluorescence images were acquired by an inverted DMI 6000 confocal scanner microscope TCS SP5 (Leica Microsystems CMS GmbH) with a 40 and 63X oil immersion objective. Images were acquired using Leica application suite advanced fluorescence software, from three chosen field. Results were confirmed by three independent experiments.

As shown in Fig. 7, 100 nM CHX prevented cell death in both Tat-stimulated neuroblastoma cells. These results demonstrate that ROS generation in Tat-stimulated cells was dependent on SMO/APAO activity. Moreover, the specific inhibition of these enzymes was able to prevent Tat-induced cell death, ultimately supporting the involvement of polyamine metabolism in Tat-induced neurotoxicity.

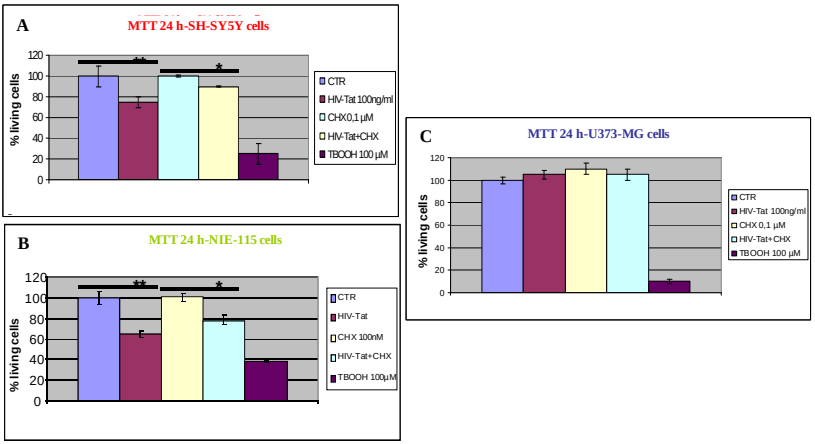

Fig.7: CHX inhibition of Tat-dependent neurotoxicity. MTT vitality assay. SH-SY5Y (**A**), U373-MG (**C**), and differentiated NIE-115 (**B**). Cells were pre-treated with 100 nM CHX overnight. 100 ng/ml HIV-1- Tat recombinant protein was added and MTT assay was performed after 24-h treatments. CHX pre-treatment demonstrate polyamine metabolism toxicity-dependent cell death. 100 µM t-BOOH treatment was performed as ROS-dependent cell death control. Data were expressed as means ± S.D of three different experiments, each with two replicates; *p<0.01, **p<0.001.

Involvement of NMDA receptor in Tat-induced cell death

In order to delve into the molecular mechanisms involved in Tat-induced neurotoxicity we assessed, firstly, the viability of SH-SY5Y and N1E-115 neuroblastoma cells after 24-h treatment with NMDA agonist in the absence or presence of NAC. As expected, we observed that the stimulation of NMDA receptor decreased significantly the cell viability in both cell lines (Fig. 8).

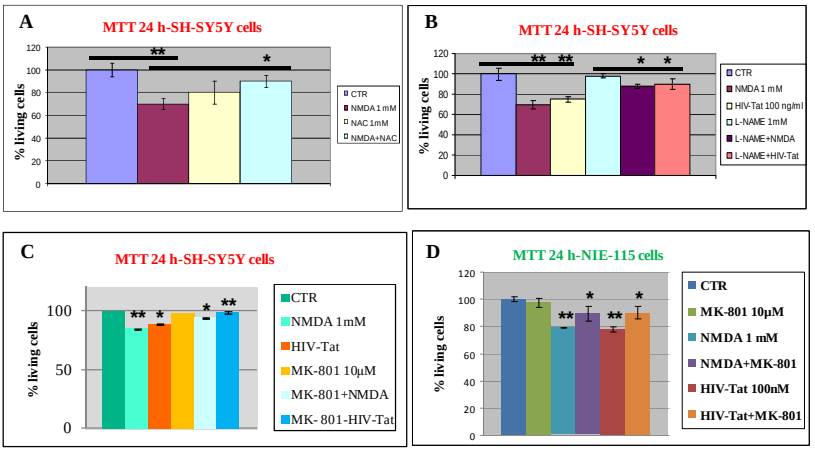

Fig.8: **L-NAME and MK-801 inhibition of Tat- and NMDA-dependent neurotoxicity. MTT vitality assay**. SH-SY5Y (**A,B,C**) and differentiated NIE-115 (**D**) cells were pre-treated with 2 mM NAC, 10 µM MK-801 or 1 mM L-NAME for 3 h. MTT assay was performed after 24-h treatments with 100 ng/ml HIV-1- Tat or 1 mM NMDA. Data were expressed as means ± S.D of three different experiments, each with two replicates; CTR vs. Treatment = **p*<0.01, ***p*<0.001.

Secondly, the involvement of NMDA receptor activation in Tat-induced cell death was demonstrated by the effect of MK-801, able to restore cell viability of both cell lines (Fig. 8 panel C and D). Results in panel B show that NMDA and Tat-induced cell death was mediated at least in part by NO production, since the pre-treatment with L-NAME restored cell viability. As shown in panel A, we observed that the decreased cell viability induced by NMDA activation, was ROS dependent since the pre-treatment with NAC was able to completely prevent this effect in agreement to ROS decrease (fig. 3 of supplement). Next, to investigate whether the production of ROS induced by Tat could be downstream the activation of NMDA receptor we

pretreated Tat-stimulated neuroblastoma cells with MK-801. We observed that overnight pre-treatment of Tat-stimulated cells with MK-801 completely prevented ROS generation (Fig. 9; fig. 4 of supplement). Also the pre-treatment with L-NAME was able to prevent Tat- as well as NMDA-induced ROS increase thus suggesting a role for NO in eliciting a further induction of ROS production.

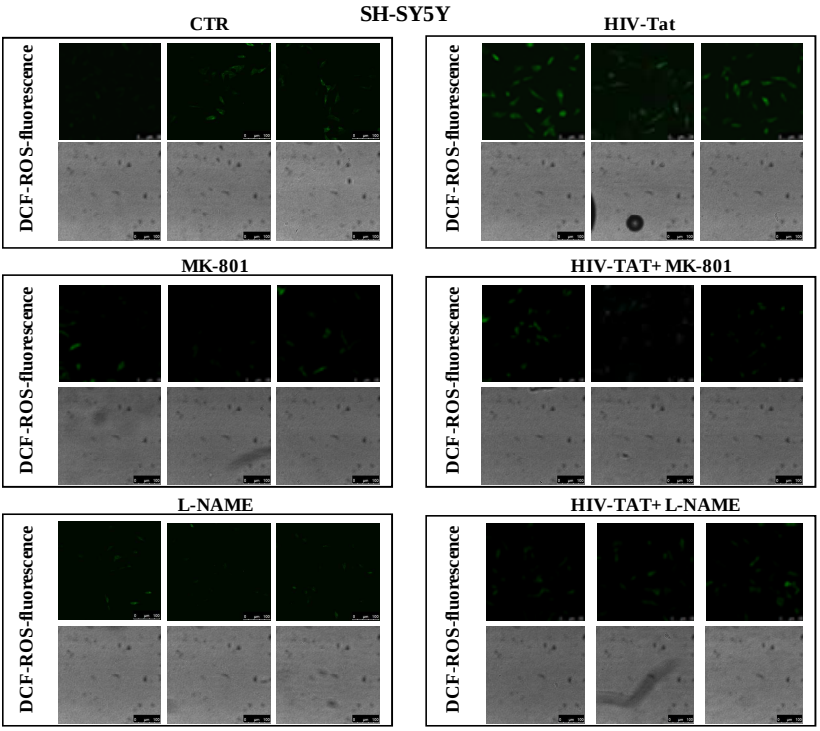

Fig.9: **NMDAR and NO involvement in HIV-Tat-dependent ROS increase.** (100 ng/ml) HIV-1 Tat-induced accumulation of intracellular ROS, already after 1-h treatments, was prevented by a 3-h preincubation of SH-SY5Y with 10 µM MK-801 and 1mM L-NAME. ROS were measured using DCF-DA fluorescence probe. Fluorescence images were acquired by an inverted DMI 6000 confocal scanner microscope TCS SP5 (Leica Microsystems CMS GmbH) with a 40 and 63X oil immersion objective. Images were acquired using Leica application suite advanced fluorescence software, from three chosen field. Results were confirmed by three independent experiments.

Finally, to explore a possible interplay between NMDA receptor activation and polyamine oxidases-mediated ROS generation we performed some preliminary experiments to measure ROS levels in NMDA-treated cells in the absence or presence of the SMO inhibitor CHX. As shown in Fig.10, the pre-treatment with CHX completely abolished NMDA-induced ROS generation, thus providing a link between polyamine metabolism and NMDA receptor stimulation.

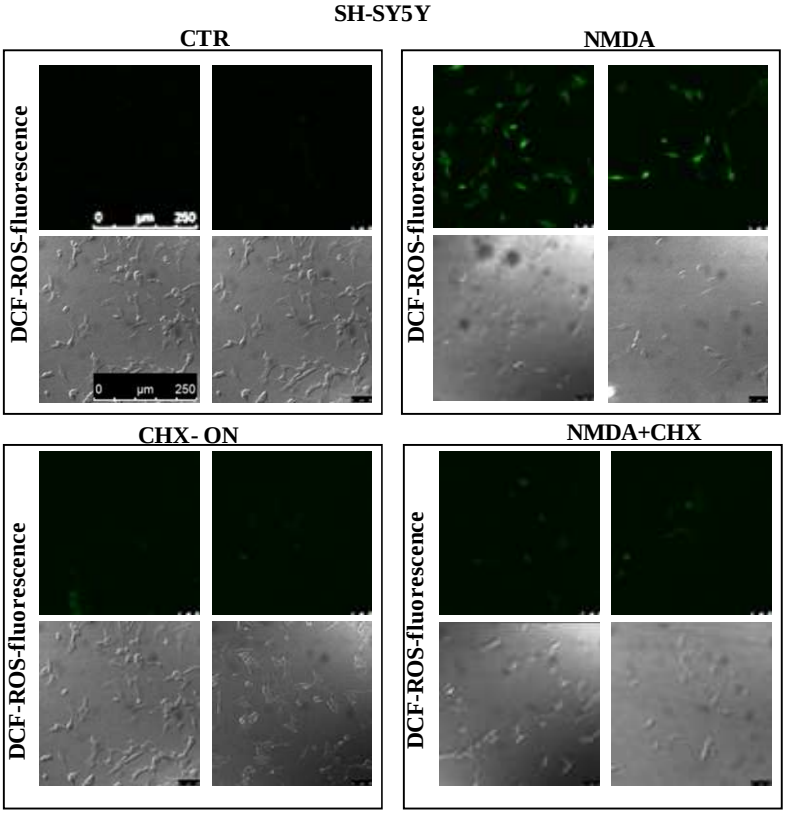

Fig.10: NMDAR involvement in ROS increase PA-oxidases mediated. 1 mM NMDA-dependent ROS accumulation was prevented by a preincubation with 100 nM CHX in SH-SY5Y. ROS were measured using DCF-DA fluorescence probe. Fluorescence images were acquired by an inverted DMI 6000 confocal scanner microscope TCS SP5 (Leica Microsystems CMS GmbH) with a 40 and 63X oil immersion objective. Images were acquired, using Leica application suite advanced fluorescence software, from three chosen field. Results were confirmed by three independent experiments.

Decrease of the intracellular glutathione (GSH) levels in neuroblastoma cells treated with HIV-Tat

Oxidative stress is also defined as an imbalance between the pro-oxidant (e.g., ROS generation) and the anti-oxidant systems (e.g., intracellular GSH e cysteine), with the shift towards the pro-oxidant system.
HIV is known to progressively deplete GSH content in patients. In our cell culture model we found that Tat treatment for 16 h caused a significant decrease of GSH levels only in neuroblastoma cells. In particular, we observed a strong reduction of ratio in Tat-treated SH-SY5Y cells (fig. 11 A, C), whereas in astrocytoma cells total GSH levels and the ratio GSH/GSSG correspond to untreated cells (fig. 11 B, D).

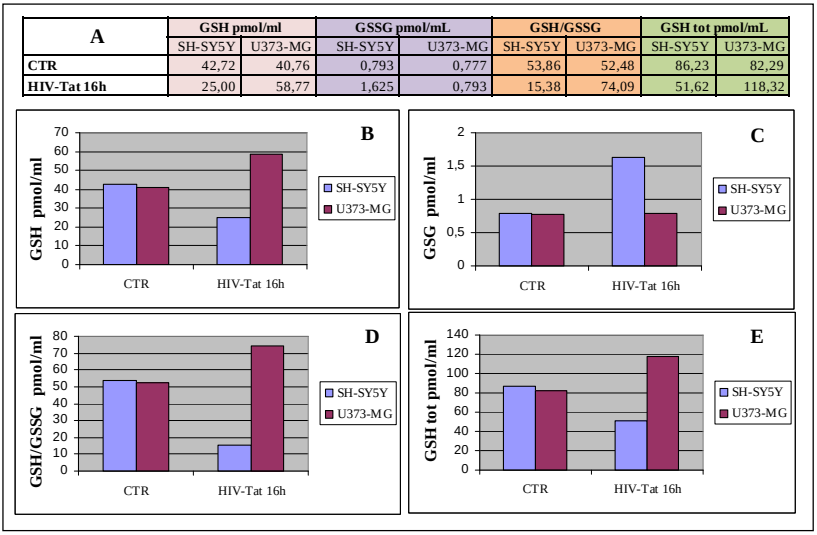

Fig.11: intracellular glutathione (GSH) levels measurement in neuroblastoma and glioma cells. (100 ng/ml) HIV-1 Tat-induced accumulation of intracellular ROS caused, after 16-h treatments, GSH/GSSG decrease in human neuroblastoma cell line (**A,D**). GSH content was increased in U373-MG treated with Tat (**A,B,E**). Measurement of GSSG content in U373-MG and SH-SY5Y cells (**C**). HPLC separation analysis was performed and data were expressed as pmol/ml.

These results are in agreement with evidence in the literature indicating that the antioxidant response occurs preferentially in astrocytes (Reddy *et al*., 2011; Kraft *et al*., 2004).

Activation of the antioxidant cell response: Nrf2 nuclear traslocation in Tat-stimulated cells

In the cell, a fundamental antioxidant defence system is represented by the induction of cytoprotective (phase-2) enzymes, such as glutathione transferases (GST), glutamate cysteine lygase (GCL), and heme oxygenase-1 (HO-1). Under basal conditions, these enzymes are present as a fraction of their full capacity, but the transcription of their cognate genes can be co-ordinately up-regulated by the Nrf2 transcription factor following exposure to a variety of stimuli, including oxidants (Motohashi et Yamamoto , 2004). In order to evaluate the activation of Nrf2 by HIV-1 Tat, U373-MG astrocytoma and SH-SY5Y neuroblastoma cells were treated with Tat (100 ng/ml) at different time points and the translocation of Nrf2 into the cell nucleus was analyzed by the TransAM assay. As shown in Fig. 11, we found Nrf2 accumulation in nuclear extracts of both Tat-treated cell lines as early as 15 min. Notably, in neuroblastoma cells we observed Nrf2 nuclear accumulation still at 4 h post-treatment.

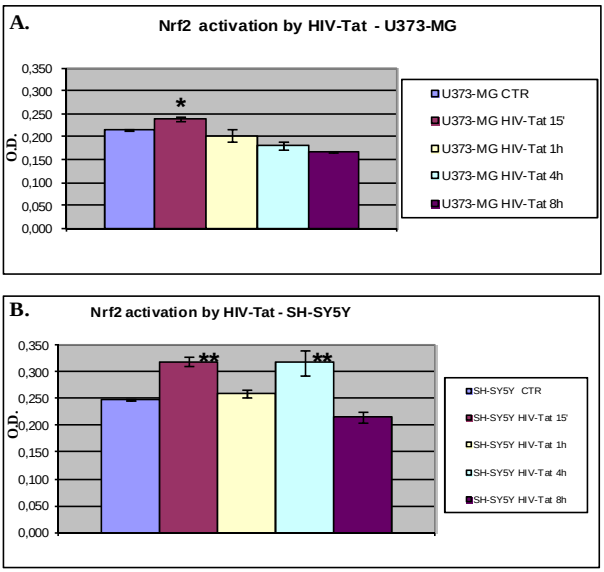

Fig. 11: TransAM assay. Nrf2 activation by HIV-1 Tat in human glioblastoma **(A)** and neuroblastoma **(B)** cells**.** Data were expressed as means ± S.D, each with triplicates. CTR vs. Treatment = *p<0.01, **p<0.001.

Activation of the antioxidant cell response: analysis of ARE-driven gene expression in Tat-stimulated cells

The activation of Nrf2 transcription factor is a condition required for the expression of ARE genes involved in cell antioxidant response. To study the expression of ARE-driven genes we performed RT-PCR analysis on total RNA extracted from Tat-stimulated cells at different time points. In particular, we evaluated the mRNA levels of the phase-2 enzymes glutamate cysteine lygase (GCL-C: catalytic subunit; GCL-M: modulatory subunit) and heme oxygenase-1 (HO-1) in astrocytoma and neuroblastoma cells treated with Tat (100 ng/ml) for 1, 4, 6, 8 h.

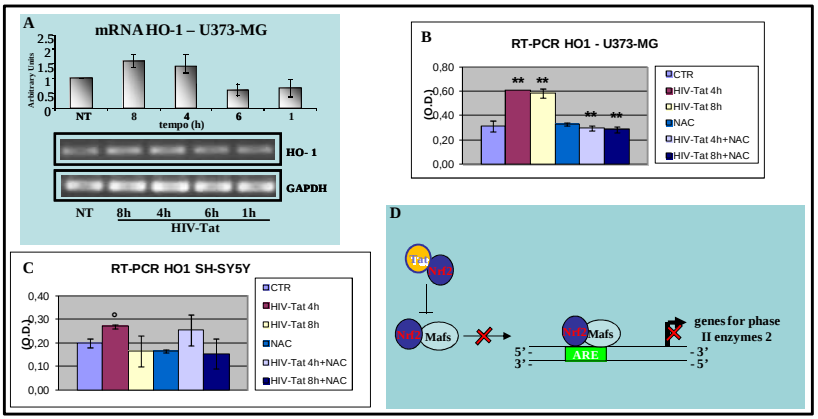

Fig. 12: NRF2/ARE pathway activation in glioblastoma and neuroblastoma cells: HO-1. (**A,B**) U373-MG and SH-SY5Y (**C**) cells were treated with 100 ng/ml HIV-Tat for 1,4, 6 and 8 h. (A,B,C). Pre-treatment with 2 mM NAC was also performed. By RT-PCR HO-1 mRNA content was determined. Data were expressed as means ± S.D. Results were confirmed by three independent experiments each with two replicates. CTR vs. Treatment = **p*<0.01, ***p*<0.001, °p=not significant. (**D**) Scheme: proposed mechanism for Nrf2 inhibition in neuroblastoma cells.

We observed that HO-1 (fig. 12 A, B) and GCL-C (fig. 13 A) were significantly upregulated in astrocytes at 4- 8-h after treatment. Interestingly, neuroblastoma cells were unable, in the same conditions, to upregulate neither HO-1 nor GCL (fig. 12 C; 13 C, D). These results are to some extent unexpected, considering that also in neuroblastoma cells Nrf2 was activated by Tat (see Fig. 11). Furthermore, astrocytoma cells pre-treated overnight with 1 mM NAC prevented HO-1 and GCL mRNA

expression highlighting the involvement of ROS as key molecular player in this pathway (Fig. 12 B; 13 A).

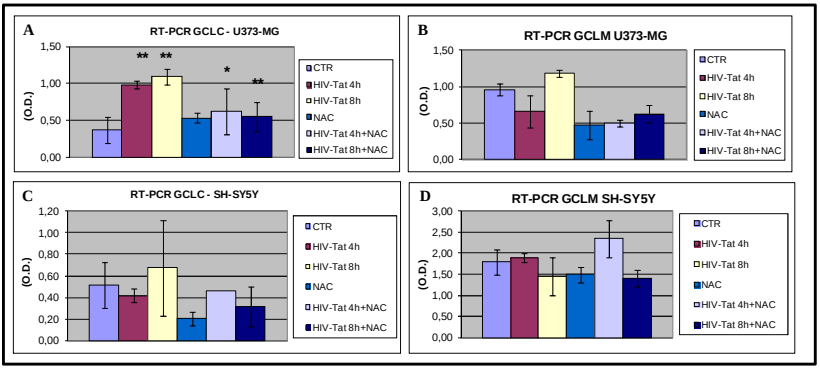

Fig. 13: NRF2/ARE pathway activation in glioblastoma and neuroblastoma cells: GCL-C and GCL-M. U373-MG (**A,B**) and SH-SY5Y (**C,D**) cells were treated with 100 ng/ml HIV-Tat for 4 and 8 h. Pre-treatment with 2 mM NAC was also performed. By RT-PCR GCL-C and GCL-M mRNA content was determined. Data were expressed as means ± S.D, each with two replicates. CTR vs. Treatment = *p<0.01, **p<0.001. Results were confirmed by three independent experiments.

The interaction of Tat with host proteins might interfere with the antioxidant response activation

In neuroblastoma cells we observed that Tat failed to induce ARE-driven gene expression in spite of Nrf2 activation. This apparent paradox could be explained by putative interaction of Tat with host proteins involved in the antioxidant response machinery. In this respect it has been recently reported that Nrf2 can interfere with the activity of Tat in inducing HIV-1 LTR transactivation (Zhang *et al.*, 2009). Thus we hypothesized that Tat might down-regulate directly the expression of Nrf2-dependent phase 2 genes through a direct interaction with Nrf2 or Nrf2-keap-1 complex. To evaluate a direct interaction between Tat and Nrf2 we used transiently transfected cells expressing Tat under the control of CMV promoter. Firstly, we cloned into the mammalian expression vector pCDNA-3.1 the HIV-1 Tat 86 aa sequence. The in frame integration of the recombinant gene was controlled by sequencing and digestion with the restriction enzymes (fig. 14 A).

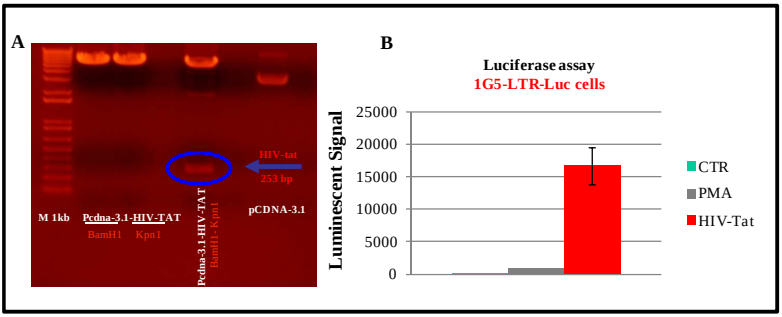

Fig. 14: HIV-1 Tat cloning and luciferase assay. **(A)** PCR: HIV-1 Tat 86 aa was cloned in pCDNA-3.1 vector between BamH1 and Kpn1 restriction sites. **(B)** 1G5/LTR-luciferase cell clone was transfected with pCDNA-3.1-HIV-Tat: Tat expression and activity were verified by luciferase assay.

The activity of the recombinant protein was verified, using the stable 1G5/LTR-luciferase Jurkat cell line-derived clone, by luciferase assay (fig.14B).
Successively we performed experiments of immunolocalization and co-immunoprecipitation with Tat-transfected cells.
By confocal fluorescence microscopy we carried out co-localization experiments to analyze the putative interaction between Nrf2 and Tat proteins.

To increase the expression of Nrf2, transfected cells were treated with 10µM Sulforaphane.
As shown in Fig.15 and 16, we observed in both cell lines a cytosolic co-localization of the two proteins.

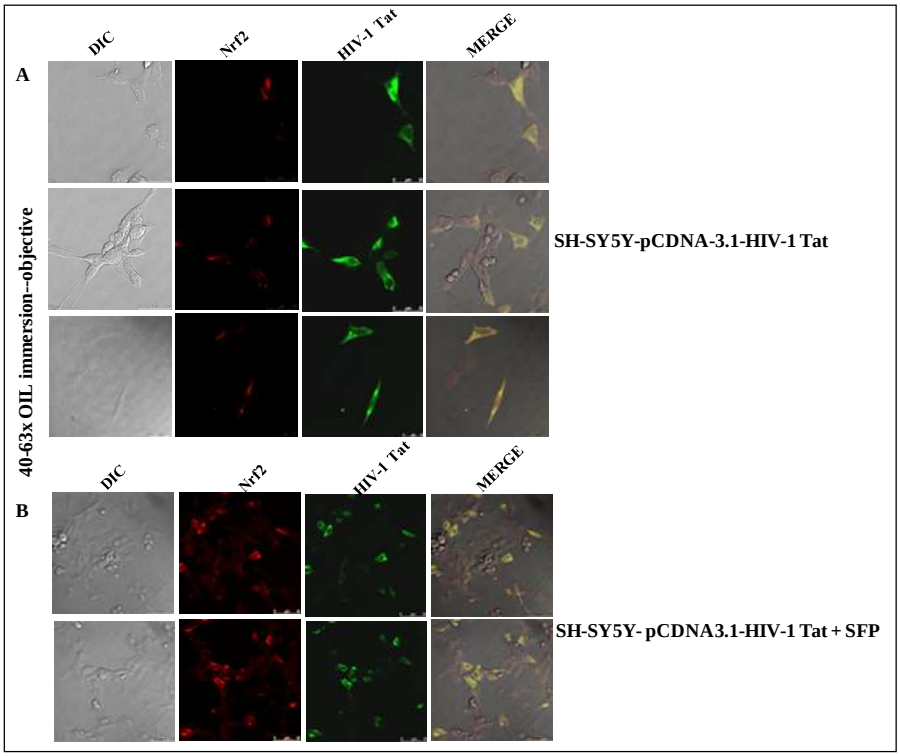

Fig. 15: NRF2/HIV-1 Tat co-localization in human neuroblastoma cells: a confocal microscopy analysis. HIV-1 Tat (green) and Nrf2 (red) immunofluorescences were respectively detected by fluorescein isothiocyanate-conjugated and red Alexafluor 594-conjugated antibodies. Overnight treatment with 10 µM SFP was performed to increase Nrf2 cell levels. Merge images (yellow) indicates that the two proteins co-localization. Results were confirmed by three independent experiments.

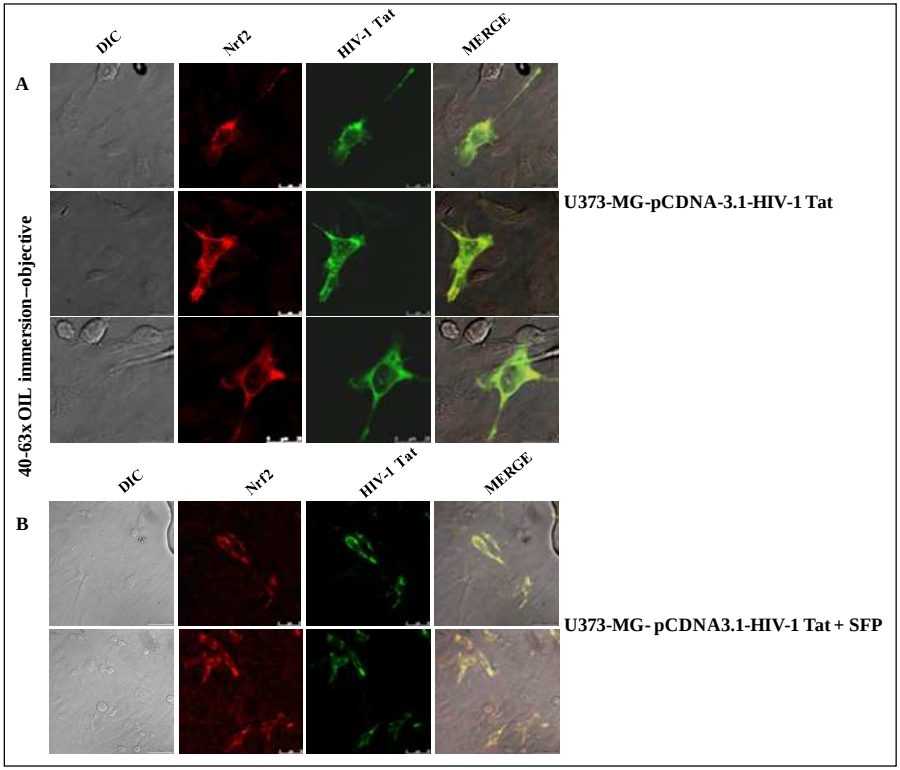

Fig. 16: Nrf2/HIV-1 Tat co-localization in human glioblastoma cells: a confocal microscopy analysis. HIV-1 Tat (green) and Nrf2 (red) immunofluorescences were respectively detected by fluorescein isothiocyanate-conjugated and red Alexafluor 594-conjugated antibodies. Overnight treatment with 10 µM SFP was performed to increase Nrf2 cell levels. Merge images (yellow) indicates that the two proteins co-localization. Results were confirmed by three independent experiments.

To better investigate on possible physical interaction between the two proteins we performed co-immunoprecipitation and Western Blot analysis on total extracts of both SH-SY5Y and U373-MG transfected cell lines. Results in figure 17 (panel A) demonstrate that the interaction between Nrf2 and Tat proteins occurred almost exclusively in SH-SY5Y.

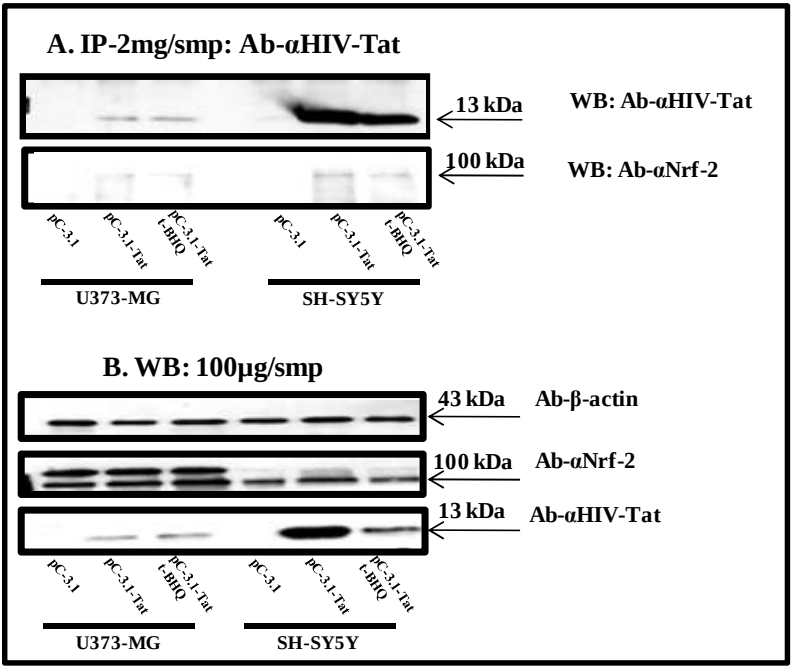

Fig. 17: IP assay for HIV-Tat/Nrf-2 interaction. $1x10^6$ SH-SY5Y and U373-MG human cells were transfected by nucleofector and lysed 24 h after transfection. Treatment with 50 µM t-BHQ was performed to increase translocation of Nrf2 into the nucleus from cytoplasm. **A**) 2mg of total extract for each sample was employed for IP assay. (**B**) 100µg of total extract for each sample was analyzed by Western Blot analysis.

Moreover we observed very higher levels of Tat in neuroblastoma with respect to astrocytoma cell extracts. This discrepancy could not be due to a different transfection efficiency between the two cell lines but rather to the ability of astrocytes to secrete Tat protein more efficiently than neurons **(**Chauhan *et al*. 2003). In support of this, we found that the supernatant of transfected astrocytoma cells was able to induce cell death unlike the supernatant derived from neuroblastoma cells (Fig. 19). Further experiments are needed to better elucidate this topic.

We also found in western blot of total cell extracts an additional band, of about 150-200 kDa, only in glial cell line (fig. 17 B).

These results suggest that in astrocytes a fraction of Nrf2 might be protected from interaction with Tat because of the formation of a complex with other host proteins. In this regard, several post-translational modification of Nrf2,

have been reported in the literature such as phosphorylation (Pi *et al.* 2007), covalent binding of an actin monomer (43 kDa) (Kang *et al.* 2002) or tetra-ubiquitin conjugation (Li *et al.* 2005).
Our results though preliminary may suggest that Tat can interact directly with a not-complexed form of Nrf2 (100 kDa) as seems to occur in neuroblastoma cells.

Supernatants of astrocytoma cells overexpressing HIV-1 Tat protein caused neuronal cell death

To verify whether HIV Tat protein could be differently secreted by glial and neuronal cells, mimicking the conditions actually occurring during brain infection, we used the supernatant of Tat-transfected neuroblastoma or astrocytoma cells to assess the neurotoxicity of the released Tat on neuroblastoma cells. Supernatants from SH-SY5Y and U373-MG cells transiently transfected with pCDNA-3.1-HIV-Tat (containing the viral HIV-1 Tat cDNA) and with the empty vector pCDNA-3.1 (as control), were tested for their ability to induce cell death. As shown in Fig. 19, the supernatant of Tat-transfected astrocytoma cells induced a significant cell death in both SH-SY5Y and NIE-115 neuroblastoma cell lines (panel B and C, respectively).

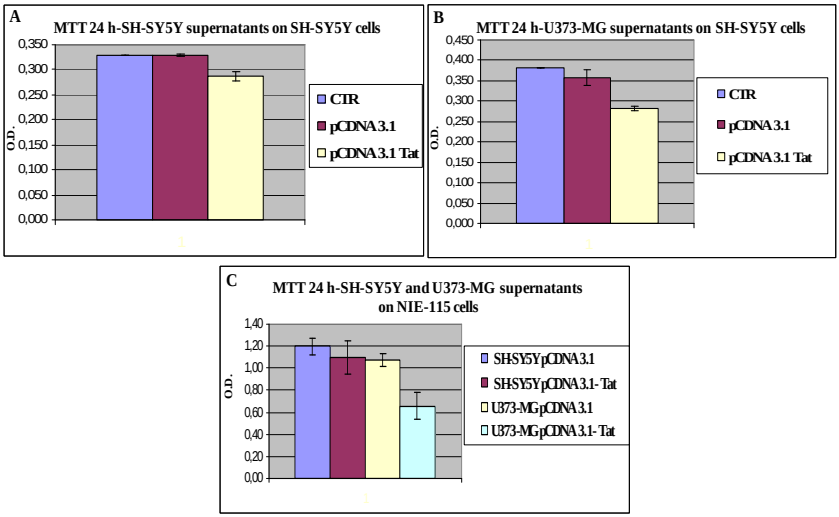

Fig. 19: Tat secreted by astrocytes is toxic for neurons. MTT assay: SH-SY5Y and U373-MG cells were transfected with pCDNA3.1-HIV-1 Tat construct and the supernatants were transferred to human (**A,B**) and murine differentiated (**C**) neuroblastoma cells, 24 h after transfection. Data were expressed as means ± S.D, each with three replicates.

The supernatant derived from Tat-transfected neuroblastoma cells was also able to induce cell death, though at a lower percentage (panel A).

Astrocytoma cells cultured with Tat-transfected cell supernatants were completely resistant to cell death (data not shown).
These preliminary data indicate that glial cells are apparently able to secrete Tat into supernatants thus leading to neuronal cell death. On the contrary, neurons are unable to eliminate the viral protein that accumulates in the cytoplasm of cells, as shown fig. 17 A by IP assay, thus increasing its toxic effect likely interfering with the cell antioxidant response machinery. Further experiments with anti-Tat neutralizing antibodies will be carried out to evaluate the specificity of secreted Tat in inducing neuronal cytotoxicity.

4. DISCUSSION AND CONCLUSIONS

HAD is a neuro-inflammatory condition characterized by the presence of HIV-infected microglial cells, astrogliosis, myelin loss and neurodegeneration. The mechanisms leading to cognitive impairment and dementia in AIDS patients are complex: various line of evidence indicate that when HIV-infected monocytes/macrophages activate neuro-inflammatory cells such as microglia and astrocytes, these cells produce chemokines, cytokines and neurotoxins that, in conjunction with secreted HIV proteins, damage neurons, leading to neuronal dysfunction and cell death probably via apoptosis. In patients with HIV-1 infection, significant neuronal loss and dysfunction occurs even though neurons are rarely infected (Masliah *et al.*, 1996; Mattson *et al*., 2005). The most commonly infected cell types in brain are microglia, macrophages, and to some extent astrocytes, although limited viral replication is produced in astrocytes. Astrocytes may serve as a reservoir for the virus and may induce neuronal damage by releasing cellular and viral products or by loss of neuronal support functions. In HIV-infected astrocytes, the regulatory gene tat is overexpressed (Nath *et al*., 2002) and mRNA levels for Tat are elevated in brain extracts from individuals with HIV-1 dementia. The mechanisms leading to neurodegeneration in HAD might involve a variety of pathways including excitotoxicity and oxidative stress. The protein Tat can induce oxidative stress in uninfected cell types through the release of nitric oxide and reactive oxygen species (Mattson *et al*., 2005).

Our results give a further contribute to the comprehension of the mechanisms involved in neurodegeneration induced by HIV.

In particular, we found that HIV-1-Tat can induce oxidative stress and neuronal cell death through the production of H_2O_2 by a mechanism involving both polyamine metabolism and NMDA receptor activation. Exogenous Tat was able to induce ROS generation in both astroglial and neuronal cell lines, leading to cell death only in neuronal cells. Moreover, our results strongly suggest that the origin of ROS generation could be related to spermine/spermidine metabolism. Indeed, in neuroblastoma cells Tat was able to induce the activity of SMO as well as the upregulation of SMO and APAO, the two enzymes involved in the metabolism of these polyamine. We provided also clearly evidence of the involvement of polyamine-derived hydrogen peroxide in Tat induced neuronal cell death since the specific inhibition of SMO/APAO completely restored cell viability. In regard to the involvement of the NMDA receptor in this

pathway we demonstrated that inhibiting polyamine metabolism by specific inhibitor completely prevented NMDA-induced cell death as well as ROS production. Finally we highlighted also the role of nitric oxide in inducing cell death as the pre-treatment of cells with the NOS inhibitor L-NAME restored cell viability even in the presence of ROS, thus suggesting that the neurotoxic effect of Tat is probably due to the concomitant NO production (i.e., peroxinitrite formation). Altogether our findings provided strong evidence that Tat can induce ROS production as a result of the interplay between NMDA receptor and polyamine metabolism. In conclusion, we propose a new pathway, involving NMDAR and SMO, as a possible mechanism for Tat-induced oxidative stress in neuronal cells, giving a possible explanation for selective death of neurons.

In the second part of this work, we focused on the activation of the antioxidant cell response in astrocytoma and neuroblastoma cells treated or transfected with Tat. The higher sensitivity of neurons with respect to astrocytes to Tat-induced cell death, could also be due to differences in the ability to activate the cell defence mechanisms between the two cell types. In this regard, our finding that in Tat-treated neuroblastoma cells the ratio GSH/GSSG was decreased by almost four times, is indicative of chronic oxidative stress and suggests the lack of an efficient antioxidant cell response.

The activation of Nrf2 transcription factor is a condition required for the expression of ARE genes involved in cell antioxidant response. A growing body of evidence indicates the Nrf2/ARE pathway as a fundamental player in the neuroprotection against oxidative stress underlining an important role for astrocytes (Johnson *et al*., 2002). Furthermore, Nrf2-dependent antioxidant response has been recently reported to be activated in astrocyte cultures infected with ts1, a retrovirus that causes a progressive neurodegenerative disease resembling to HIV-associated dementia (Qiang *et al.*, 2006). In order to analyze and to compare the antioxidant cell response in astroglial and neuronal cell lines, we investigated whether Tat expression could be implicated in Nrf2 activation and in ARE-driven gene expression. The results obtained demonstrate that although Nrf2 was activated by Tat in both cell lines, the expression of the Nrf2-dependent phase-2 genes was upregulated exclusively in astroglial cells. Our data clearly show the inability of neuroblastoma cells to activate an efficient antioxidant response even in the presence of activated Nrf2. In order to explain paradox we hypothesized a putative interaction of Tat with host proteins involved in the antioxidant response machinery that should lead to decreased transcription of Nrf2-dependent genes. In this respect it has been

recently reported that Nrf2 can interfere with the activity of Tat in inducing HIV-1 LTR transactivation (Zhang *et al.*, 2009). We found that Nrf2 was expressed in both cell lines at equivalent levels although in astrocytoma cells Nrf2 would seem to be associated to other host proteins thus protecting it from interaction with Tat. Moreover, our preliminary data indicate a different ability of astroglial and neuronal cell lines in releasing Tat, that could result in the accumulation of the viral protein within neuronal cells thus increasing its toxic effect, likely interfering with the cell antioxidant response machinery.

On the contrary astrocytes, being able to release the viral protein into the extracellular space, can activate the antioxidant pro-survival response. The obtained results suggest a mechanism by which Tat could modulate the antioxidant response in neurons thus leading to cell death.

However, additional studies will be required to obtain a detailed understanding of the specific proteins involved in regulation of this pathway.

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APPENDIX

Publications during Ph.D.:

Fabrizio Manetti, Alessandra Cona, Lucilla Angeli, Claudia Mugnaini, Francesco Raffi, **Caterina Capone**, Elena Dreassi, Alessandra Tania Zizzari, Alessandra Tisi, Rodolfo Federico, Maurizio Botta. Synthesis and biological evaluation of guanidino compounds endowed with subnanomolar affinity as competitive inhibitors of maize polyamine oxidase. Journal of Medicinal Chemistry. 2009 Aug 13;52(15):4774-85.

Paper submitted during Ph.D.:

Tiziana Persichini; Giovanni De Francesco; **Caterina Capone**; Maria Carmela Bonaccorsi di Patti; Marco Colasanti; G. Musci. Reactive oxygen species are involved in ferroportin degradation induced by ceruloplasmin mutant Arg701Trp. Journal of Neurological Science, Submitted October 2011.

Manuscript in preparation during Ph.D.:

Title: MOLECULAR MECHANISMS OF HIV TAT-INDUCED NEURONAL TOXICITY: A ROLE FOR POLYAMINE OXIDASES AS MEDIATORS OF ROS PRODUCTION".

ABREVIATION INDEX:

Alzheimer's disease (**AD**); Amyotrophic Lateral Sclerosis (**ALS**); Antioxidant Responsive Elements (**ARE**); Brain Microvascular Endothelial Cells (**BMVEC**); Central Nervous System (**CNS**); Chlorhexidine (**CHX**); c-Jun N-terminal kinase **(JNK)**; Dichlorofluorescein diacetate (**CM-H_2DCF-DA**); Dizocilpine (**MK-801**); g-glutamate–cysteine–glycine (**GSH**); Glutamate cysteine lygase (**GCL**); Glutamate cysteine lygase-catalitic subunit (**GCLC**); Glutamate cysteine lygase-modulatory subunit (**GCLM**); Glutathione transferases (**GST**); Heme oxygenase-1 (**HO-1**); HIV-associated dementia (**HAD**); HIV-associated neurocognitive disorders (**HAND**); Human Immunodeficiency Virus (HIV); Huntington's disease (**HD**); Kelch-like ECH-associated protein-1 (**KEAP1**); L-Nitro-Arginine Methyl Este (**L-NMAE**); Long Terminal repeats (**LTR**); N1-acetyl polyamine oxidase (**APAO**); N-Acetyl-l-cysteine (**NAC**); NF-E2 related factor-2 (**Nrf2**); Nitric Oxide (**NO**); Nitric Oxide Synthases (**NOS**); N-methyl-D-aspartate receptor (**NMDAR**); Oxidised glutathione (**GSSG**); Parkinson disease (**PD**); Quinone Oxidoreductase (**NQO1**); Reactive Nitrogen Species (**RNS**); Reactive Oxygen Species (**ROS**); Reduced glutathione (**GSH**); spermine oxidase (**SMO**); Sulforaphane (**SFP**); tert-butyl hydroperoxide (**t-BOOH**); tert-butylhydroquinone (**t-BHQ**); Transcription Factor Activator Protein (**AP-1**); Transcriptional Transactivator protein (**Tat**); viral protein R (**Vpr**).