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Attenuated neurotoxicity of the transactivation-defective HIV-1 Tat protein in hippocampal cell cultures

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In the era of highly-effective anti-retroviral therapy (HAART) neurological disorders linked to HIV-1 infection have become a major source of morbidity in AIDS (Navia et al., 1986; Koultsilieri et al., 2002; Manji and Miller, 2004). HIV-1 virus does not infect neurons. HIV-associated neurodegenerative pathology is widespread and is not proportional to viral load. Therefore, the neurotoxic potential of different HIV proteins attracts significant attention (Van de Bovenkamp et al., 2002; Jones and Power, 2006; Hult et al., 2008). Although mechanisms that incite neurodegeneration in AIDS are likely to be multifactorial, viral proteins appear to contribute to increased neuronal apoptosis in the HIV-infected brain (Roshal et al., 2001; Cossarizza, 2008).

The typical HIV genome has three major coding regions: core (gag); polymerase (pol); and envelope (env). The HIV genome also encodes ‘accessory’ or ‘regulatory’ proteins such as Nef, Tat, Vpr, Vif, Rev and Vpu that play a key role in the pathogenesis of HIV infection (Ozdener, 2005; Hult et al., 2008). The Tat protein is a key HIV-1 transactivator, which is essential for the control of HIV-1 gene transcription. The full-length open reading frame of HIV-1 Tat consists of the two exons of the viral *tat* gene and encodes a protein of approximately 101 amino acids (Hetzer et al., 2005). HIV infected cells excrete Tat, and extracellular Tat can be then taken up by non-infected cells (Ensoli et al., 1993; Tardieu et al., 1992; Chang et al., 1997). Due to the intrinsic ability of Tat to interact with various cell components, this regulatory HIV protein can interfere with a variety of biochemical processes (Shojania and O’Neil, 2006). HIV-1 Tat is proven to be neurotoxic. Existing evidence for direct and indirect neurotoxicity of Tat rationalizes suggestions for an important role of this regulatory HIV protein in the pathogenesis of NeuroAIDS and stimulates the investigation of molecular mechanisms of Tat interactions with brain cells (King et al., 2006). Molecular determinants of Tat neurotoxicity are located within the 1–72 sequence encoded by the first *tat* exon. The second exon-encoded part of the full-length Tat sequence has been shown to be non-essential for the direct neurotoxicity (Nath et al., 1996). Amino acid residues 22–38 comprise highly conservative cysteine-rich domain of Tat. Experimental evidence, which indicates the important role of the cysteine-rich domain in the mechanisms of Tat toxicity, is accumulating rapidly (Misumi et al., 2004; Egelè et al., 2008; Mishra et al., 2008). The discovery of the lower cytotoxic potential of HIV-1 virus clade C, which is thought to be attributed to the mutation of cysteine 31 in the 1–101 version of Tat protein expressed in this HIV-1 subtype (Ranga et al., 2004; Mishra et al., 2008; Li et al., 2008), has fueled the interest to the role of individual cysteines located in the cysteine-rich domain in the ability of Tat to cause neurodegeneration.

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In this study, we investigate the effect of the mutation of the cysteine 22, which is essential for Zn²⁺-chelating and trans-activation abilities of HIV-1 Tat (Saidae et al., 1990; Garber et al., 1998), with Gly on neurotoxic properties of the recombinant Tat 1–86 (clade B) in the primary culture of rat fetal hippocampal neurons.

Recombinant original Tat 1–86 and (Cys22→Gly22)-substituted Tat 1–86 (LAI/Bru strain of HIV-1 clade B, GenBank accession no. K02013) were purchased from Diatheva (Italy). Tat 1–101 clade C was purchased from Prospec (Israel).

Primary hippocampal cell cultures were prepared from 18-day-old Sprague-Dawley rat fetuses as previously described (Aksenov et al., 2006). Cultures were used for experiments after 12 days in culture and were >85–90% neuronal as determined by anti-MAP-2/anti-GFAP/Hoechst fluorescent staining.

The treatment of hippocampal cell cultures was carried out by the addition of freshly-prepared stock solutions of the recombinant Tat polypeptides into the cell culture growth medium. Equal volume of the vehicle was added to control cell cultures. To determine dose-response neurotoxicity curves, groups of individually grown cell cultures were exposed to 10–150 nM concentrations of Tat or Cys22 Tat for 48 hours. For the time course experiments, cell cultures were continuously incubated with 50 nM Tat or 50 nM Cys22 Tat for 2, 24, 48, and 96 hours. To study binding/uptake, 50nM Tat or 50 nM Cys22 Tat was added to the cultured cells and the incubation was carried out for different time periods from 1 min to 2 hours. Cytotoxic effects induced during different time periods (1 min –2 hours) of transient exposure of hippocampal cultures to Tat- or Cys22 Tat were studied as described in (Aksenova et al., 2009). For the comparison of the neurotoxic effects of Cys 22 Tat 1–86, Tat 1–86 (clade B), and Tat 1–101 (clade C), hippocampal cultures were exposed for 48 hours to 100 nM dose of either of the recombinant Tat polypeptides.

Neuronal survival was determined using a Live/Dead viability/cytotoxicity kit from Molecular Probes (Eugene, OR) in rat fetal hippocampal cell cultures prepared in 96-well plates as described in (Aksenov et al., 2006; Aksenova et al., 2009). Fluorescence was measured using a Bio-Tek Synergy HT microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Binding/uptake of Tat and Cys22 Tat was studied using anti-Tat immunocytochemistry in acetic alcohol-fixed cultures, immunoblotting of cell lysates, and the direct ELISA measurements of concentrations of Tat polypeptides in the cell culture growth medium (Aksenova et al., 2009). Rabbit polyclonal anti-Tat antibody (Diatheva, Italy) that recognizes both Tat and Cys 22 Tat immunoreactivities was used in these experiments.

Fluorochrome Inhibitor of Caspases (FLICA) Caspase 9 (red fluorescence) or Caspase 3/7 (green fluorescence) Apoptosis Detection Kit (Immunochemistry Technologies LLC, Bloomington, MN) were used to detect active caspases in cultures exposed to Tat and Cys22 Tat. Hoechst staining (blue fluorescence) was used to label cell nuclei. Microplate reader-based analysis and fluorescence microscopy imaging of specific fluorescence of SR-LEHD or FAM-DEVD complexes with active caspase 9 or 3/7 were carried out as described in (Aksenova et al., 2009).

Statistical comparisons were made using ANOVA and planned comparisons were used to determine specific treatment effects. Significant differences were set at $P < 0.05$.

The dose-response curve of Tat 1–86 toxicity shown in Figure 1A was consistent with our earlier reports (Aksenov et al., 2006; Aksenova et al., 2009). The analysis of dose-response curves demonstrated that Tat 1–86 was significantly ($P < 0.05$) more toxic to hippocampal cell cultures than Cys22 Tat 1–86 following 48 hours of treatment.

It could be argued that the substitution of cysteine 22 in Tat 1–86 simply delayed the development of Tat-mediated neurotoxicity. However, the comparison of the toxicity time courses in Tat - and Cys22 Tat- (Figure 1B) did not favor this suggestion since even the prolonged exposure to cysteine 22-substituted Tat variant failed to induce more than 10% decrease in cell viability. At 96-hour time point the Live/Dead ratio in Cys22 Tat-treated cultures was $90.7 \pm 4.43\%$ of control and this difference in cell viability did not reach statistical significance ($P > 0.05$) due to high variation of individual numbers.

Binding to the cell membrane is the key step in the process of Tat neurotoxicity (Nath et al., 1999; Chandra et al., 2005; Aksenova et al., 2009). According to the literature, the cysteine 22 mutation does not affect intracellular distribution of the extracellular Tat protein following its internalization (Sadaie, 1990). Nevertheless, it was still possible that altered binding/uptake properties could be a reason for the attenuated neurotoxic ability of Cys22-substituted Tat 1–86. The presence of cell-bound/internalized Tat 1–86 or Cys22 Tat 1–86 in hippocampal neurons after 2-hour exposure to 50 nM dose to either one of the Tat variants was evident by immunofluorescent microscopy and Western blotting (Figure 2A, B). The curves describing the specific absorption of Tat immunoreactivity by hippocampal cells in Tat 1–86- and Cys22 Tat 1–86-exposed cultures were not significantly different from each other (Figure 2C). Neurotoxic effects produced by the transient exposure of cell cultures to 50 nM Tat 1–86 were time-dependent and consistent with the kinetic of Tat absorption in hippocampal cultures (Figure 2D). In contrast, transient exposure of cultures to 50 nM Cys22 Tat 1–86 was unable to cause cytotoxicity. Thus, we conclude that the impaired toxicity of Cys22 Tat 1–86 was not attributable to any noticeable changes in its ability to interact directly with neurons.

The variant of Tat protein from HIV-1 clade C (Tat 1–101, clade C) was reported to be less neurotoxic than its analogs from HIV-1 clade B strains (Ranga et al., 2004; Mishra et al., 2008; Li et al., 2008). More than 90% of HIV-1 clade C viruses encode Tat with serine at the position 31 instead of cysteine 31 (Ranga et al., 2004). Therefore, we determined the toxicity of an equal dose of Tat clade B, Cys22-substituted Tat clade B and Tat clade C in hippocampal cell cultures. The 100 nM final concentration is the dose of recombinant Tat clade B that produces maximum cell viability decreases (Live/Dead ratio: $75 \pm 2.6\%$ of control) in hippocampal cell cultures. The Live/Dead ratios in groups of cultures treated with Cys22 Tat clade B or Tat clade C were $90 \pm 1.4\%$ vs control and $95 \pm 3.0\%$, respectively. Comparisons of cell culture groups exposed to either Cys22 Tat 1–86 or Tat 1–101 (clade C) demonstrated that both Tat variants were significantly ($P < 0.05$) less neurotoxic than Tat 1–86 (clade B). The cell viability changes induced by 48-hour treatment of cell cultures with equal doses of either Cys22 Tat 1–86 or Tat 1–101 (clade C) were not significantly different ($P > 0.05$). If indeed the attenuated neurotoxicity of HIV-1 C Tat is attributed to the conservative Cys31 mutation (Mishra et al., 2008; Li et al., 2008), our results imply that point-mutation of two different cysteine residues within the Cys-rich domain may similarly impair the ability of Tat to cause neuronal degeneration.

Extracellular Tat is known to induce caspase activation in primary cultures of rat fetal neurons (Kruman et al., 1998; Aksenova et al., 2009). Consistently, we have detected the increased activity of the initiator caspase 9 following the 2-hour exposure of hippocampal cultures to 50 nM Tat 1–86 ($129.7 \pm 4.4\%$ SR-LEHD red fluorescence vs vehicle-treated control, $n=7$). Activation of the effector caspase 3/7 ($179.8 \pm 4.5\%$ FAM-DEVD green fluorescence vs vehicle-treated control, $n=8$) was determined in hippocampal cultures after the 24-hour exposure to 50 nM Tat 1–86. Consistent with the results of the microplate reader-based measurements of caspase 9 and 3/7 activities, increased numbers of SR-LEHD and FAM-DEVD-positive cells in Tat-treated cultures were observed using fluorescent microscopy. Under the standard image acquisition conditions, 9–15 SR-LEHD- positive cells per hundred of Hoechst-labeled cells were typically found in hippocampal cultures, which were exposed

to 50 nM Tat 1–86 for 2 hours and 17–20 cells out of a hundred of Hoechst-stained cells were FAM-DEVD-positive following 24-hour Tat exposure. On the contrary, neither method of the analysis of SR-LEHD and FAM-DEVD fluorescent signals have detected increased caspase activities in cultures exposed to 50 nM Cys22 Tat (Figure 2E).

Results of this study clearly demonstrate that the mutation of cysteine 22, which is a part of the putative HIV-1 Tat metal-binding site (Frankel et al., 1988; Saidae et al., 1990; Huang and Wang, 1996), reduces the ability of the recombinant Tat 1–86 (clade B) to initiate apoptotic cascades in hippocampal neurons. Our observations are in line with the experimental evidence suggesting that zinc binding plays an important role in apoptosis induced by extracellular Tat in mammalian cells (Misumi et al., 2004; King et al., 2006; Egelé et al., 2008).

Interactions with various membrane-integrated proteins play a key role in the mechanism of neuronal degeneration induced by extracellular Tat (for the review see King et al., 2006). At the cell membrane level, cysteines involved in metal-chelating (particularly Zn²⁺-chelating) may be essential for Tat's ability to alter functioning of neuronal receptors and transporter proteins, and trigger downstream cell death signaling. There is evidence indicating that the Cys22 mutation in Tat 1–86 renders it unable to interact with the dopamine transporter and thereby inhibit dopamine uptake in dopaminergic cells (Zhu et al., 2009). It is possible that the substitution of cysteine 22 may impair the ability of Tat to interfere with the Zn²⁺-mediated control of NMDAR activity and thereby attenuate Tat pro-apoptotic effects in hippocampal neurons. An important role of Tat actions at the NMDA receptor's Zn²⁺ binding site for the ability of Tat to potentiate NMDA-induced currents was suggested in several studies (Song et al., 2003; Chandra et al., 2005). Extensive stimulation of NMDAR can trigger the intrinsic apoptotic pathway mediated by the release of mitochondrial cytochrome *c* and activation of caspases 9 and 3 (Zeron et al., 2004).

Additional studies are needed to elucidate the role of different cysteine residues from the Tat cysteine-rich domain in pathways of Tat-mediated neuronal cell death. Targeting of cysteines that form the Tat metal-binding site may be considered as one of the therapeutic strategies to simultaneously block transactivating and neurotoxic Tat abilities in NeuroAIDS.

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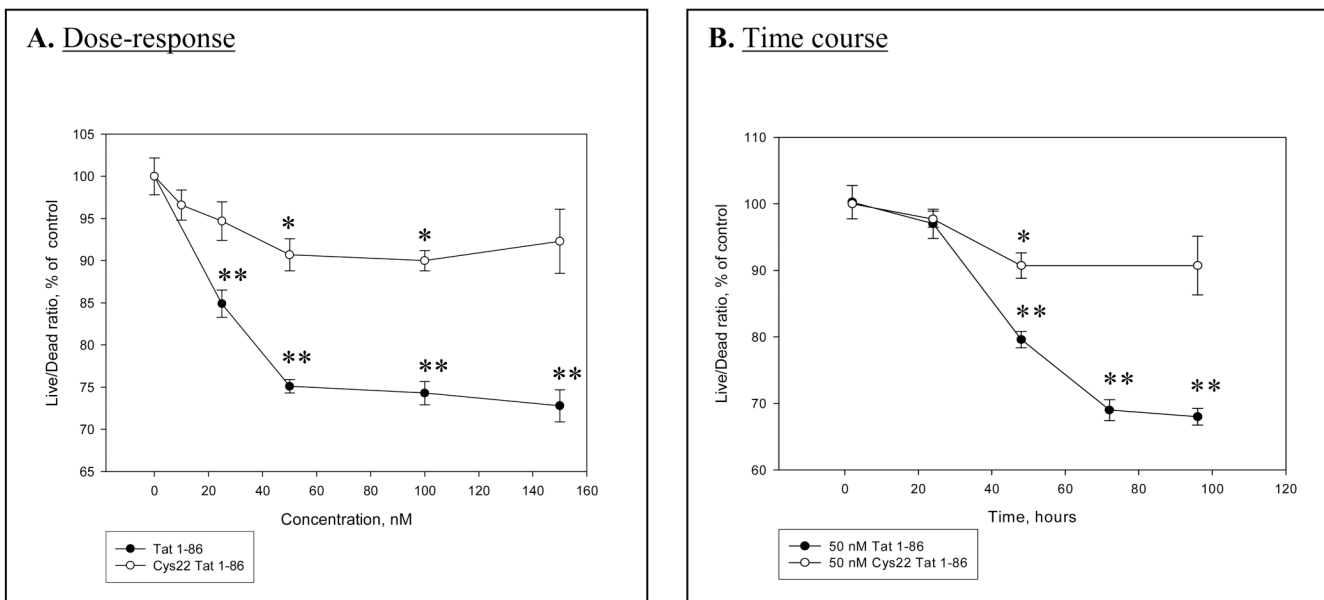


Figure 1. Neuronal cell viability changes in hippocampal cell cultures induced by the original and cysteine 22-substituted Tat 1-86 clade B

(A) The dose-response of decreased neuronal cell viability in primary rat fetal hippocampal cell cultures exposed to Tat 1-86 or Cys 22 Tat 1-86. The graph shows the decrease in Live/Dead ratios produced by different doses of recombinant Tat 1-86 after 48 hours of treatment. Data presented as mean values, n of sister cultures analyzed = 8–15 per each Tat 1-86 concentration. *- marks significant ($P < 0.05$) differences in Live/Dead ratios between cultures treated with Cys22 Tat 1-86 and vehicle-treated controls. ** - marks significant ($P < 0.05$) differences in Live/Dead ratios between cultures treated with Tat 1-86 and cultures exposed to the same dose of Cys22 Tat 1-86. **(B)** The time course of the changes in neuronal cell viability in primary rat fetal hippocampal cell cultures exposed to Tat 1-86 or Cys 22 Tat 1-86. The graph represents relative (compared to non-treated controls) changes in Live/Dead ratios following the addition of 50 nM Tat 1-86 or 50 nM Cys 22 Tat 1-86. Individual measurements were carried out in 4–8 sister cultures (wells of the 96-well plate) per each time point and the experiment was repeated three times to ensure the reproducibility of the results. Data presented as mean values. *- marks incubation time points when significant ($P < 0.05$) differences in Live/Dead ratios between cultures treated with Cys22 Tat 1-86 and vehicle-treated controls have been observed. ** - marks incubation time points when significant ($P < 0.05$) differences in Live/Dead ratios between cultures treated with Tat 1-86 and Cys22 Tat 1-86 have been observed.

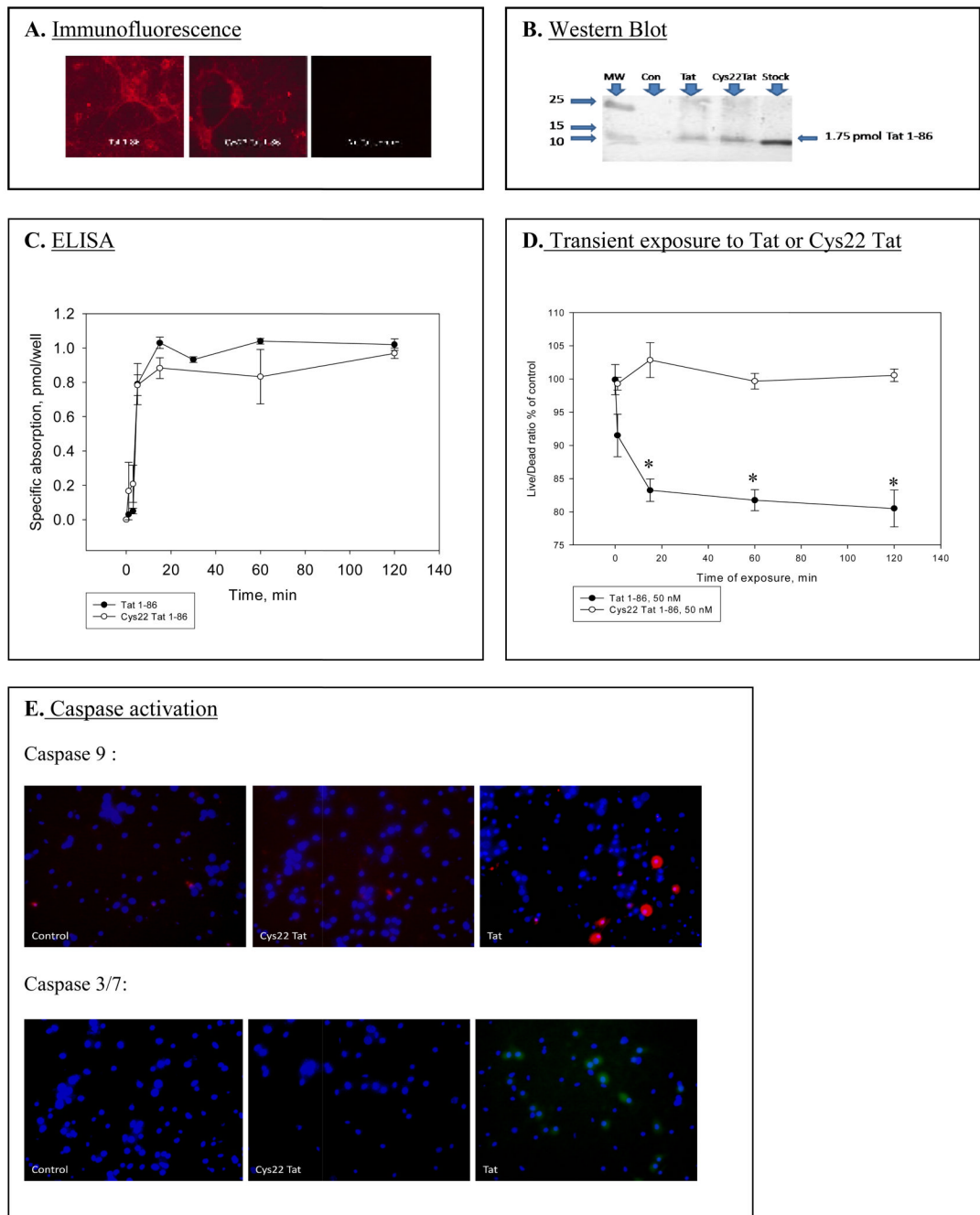


Figure 2. Direct interactions of Tat 1–86 and Cys22 Tat 1–86 with hippocampal cells and following changes in cell viability and caspase activation

Binding/uptake of Tat 1–86 or Cys22 Tat 1–86 by cultured rat fetal hippocampal neurons.

(A) Images show the specific Tat (or Cys22 Tat) immunoreactivity in rat fetal hippocampal cell cultures exposed to 50 nM Tat 1–86 or 50 nM Cys 22 Tat 1–86 for 2 hours. (B) Western blots show the specific Tat immunoreactivity in cell lysates 2 hours after the addition of either 50 nM Tat or 50 nM of Cys22 Tat 1–86 to the cell culture medium. (C) The graph shows amounts of Tat 1–86 or Cys22 Tat 1–86 per well specifically absorbed by hippocampal cells during the first 2 hours of treatment. Data presented as mean values \pm SEM (n=3 per each time point). Neurotoxic effects of the transient exposure of primary rat fetal hippocampal cell

cultures to Tat 1–86 or Cys 22 Tat 1–86. **(D)** Cell cultures were exposed to a 50 nM dose of Tat 1–86 or Cys22 Tat 1–86 for different time periods ranging from 1 min to 2 hours. After exposure, the cell culture medium was replaced with a fresh portion of medium without Tat or Cys22 Tat. The graph shows Live/Dead ratios determined in hippocampal cell cultures 48 hours after different time periods of transient exposure to original and Cys22-substituted Tat 1–86. Data presented as mean values, n of sister cultures analyzed = 7–12 per each time point. * marks time periods of transient exposure to Tat 1–86, which were sufficient to induce a significant ($P < 0.05$) decrease in Live/Dead ratios compared to vehicle-treated controls. The caspase 9 and caspase 3/7 activities in primary rat fetal hippocampal cell cultures after the exposure to Tat 1–86 or Cys 22 Tat 1–86. **(E)** Representative images show results of the detection of the SR-LEHD (red) fluorescent signal attributed to activated caspase 9 in neurons treated for 2 hours and results of the detection of the FAM- DEVD (green) fluorescent signal attributed to activated caspase 3/7 in neurons treated for 24 hours (B) with either 50 nM Tat 1–86 or 50 nM Cys 22 Tat 1–86. Cultures were co-stained with Hoechst (blue fluorescence).