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## HIV-1 protein-mediated amyloidogenesis in rat hippocampal cell cultures

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### Abstract

Since the beginning of the highly active antiretroviral therapy (HAART) era, epidemiological evidence indicates an increasing incidence of Alzheimer's (AD)-like brain pathology in aging HIV patients. Emerging evidence warns of potential convergent mechanisms underlying HIV- and A $\beta$ -mediated neurodegeneration. We found that HIV-1 Tat and gp 120 promote the secretion of A $\beta$  1–42 in primary rat fetal hippocampal cell cultures. Our results demonstrate that the variant of Tat expressed by the neurotropic subtype of HIV-1 virus (HIV-1 clade B) specifically induces both the release of amyloidogenic A $\beta$  1–42 and the accumulation of cell-bound amyloid aggregates. The results of the research rationalize testing of the ability of  $\beta$ -amyloid aggregation inhibitors to attenuate HIV protein-mediated cognitive deficits in animal models of NeuroAIDS. The long-term goal of the study is to evaluate the potential benefits of anti-amyloidogenic therapies for management of cognitive dysfunction in aging HIV-1 patients.

### Keywords

Cell Culture; HIV Tat; HIV gp120; Amyloid Peptide; Neurotoxicity

### Introduction

The development of persistent cognitive deficits in aging HIV patients with suppressed viral replication is now recognized as a serious and growing medical problem [25]. HAART medication has shifted neuropathology from a subacute encephalitic condition to a more subtle neurodegenerative process involving synaptic and dendritic degeneration, particularly of hippocampal neurons [30]. Epidemiologic evidence indicates a growing incidence of AD-like brain pathology in aging HIV patients. Clinical studies reveal similar changes in A $\beta$  1–42 levels in CSF from cognitively impaired HIV-1 patients and in patients with mild dementia of the AD-type [9]. These alarming facts suggest that HIV-associated pathology is evolving [7]. Neurotoxic regulatory and core viral proteins, such as Tat and gp120, are thought to contribute to the development of HIV-associated cognitive dysfunction. Although the role of viral proteins in HIV-associated memory impairment has been documented, the mechanism of this dysfunction is poorly defined. Recent reports warn that A $\beta$  biogenesis and clearance venues may be influenced by HIV-1 proteins [19,30].

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Adverse effects of HIV-1 Tat and gp120 on neuronal function [6,11,29] resemble alterations elicited by soluble A $\beta$  oligomers [28]. Existing evidence of strikingly similar characteristics of beta-amyloid- and HIV protein-mediated neurodegeneration rationalizes the idea that viral proteins, which are known to induce deficits in learning and memory, may facilitate accumulation of misfolded A $\beta$  in hippocampal cells. Nevertheless, the potential ability of Tat or gp120 to induce formation of amyloid aggregates has not been systematically investigated.

In this study, we investigate the connection between Tat or gp120 -mediated cell injury and amyloid formation in primary cultures of rat fetal hippocampal neurons.

## Materials and Methods

*Purified proteins.* 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). The recombinant gp120 was purchased from Protein Sciences, (Meriden, CT). Synthetic A $\beta$  1–42 was purchased from Anaspec (Fremont, CA).

*Primary hippocampal cell cultures* were prepared from 18-day-old Sprague-Dawley rat fetuses as previously described [2]. Cultures were used for experiments after 14 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 gp120, Tat polypeptides, or pre-aggregated stock solutions of A $\beta$  1–42 into the cell culture growth medium. An equal volume of the vehicle was added to control cell cultures. The preparation of pre-aggregated A $\beta$  1–42 was carried out as previously described [1].

*Hippocampal cell viability* was assessed using the microplate reader-formatted [2,3,4] variant of the Live/Dead assay (Molecular Probes, Inc., Eugene, OR). Fluorescence was measured using a Bio-Tek Synergy HT microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

*Direct ELISA measurements* of the A $\beta$  1–42 immunoreactivity in cell-conditioned medium (CM) samples were performed using the rabbit polyclonal anti-A $\beta$  1–42 antibody (Abcam Inc, Cambridge, MA, 1:500). Specificity of anti-A $\beta$  antibodies was tested using samples of fresh cell culture medium with and without recombinant HIV-1 proteins (negative controls). Serial dilutions of freshly prepared stocks of the synthetic rat A $\beta$  1–42 (0–2500 nM) with cell culture medium were used for calibration.

*The formation of cell-bound amyloid  $\beta$ -sheet aggregates* in living hippocampal cells was analyzed by fluorescent/DIC microscopy following the Congo Red staining (20  $\mu$ M Congo Red for 30 min). The Congo Red specific binding (543 nM excitation/560–615 nm emission) was visualized using a 20 $\times$  objective of the inverted fluorescent microscope (Nikon Eclipse TE2000-E). Congo Red-staining and DIC images were captured using a CCD camera. Merged images of the specific Congo Red binding and DIC were produced and analyzed by the NIS Elements imaging software (Nikon). Parts of merged Congo Red/DIC images were magnified by placing a selection box over the area of interest and saving the selection as a new image with higher resolution.

Numbers of Congo Red-positive cells were counted using the Object counting option of the NIS Elements imaging software package in 20 $\times$  images of 4 random fields of vision. For each field of vision total numbers of hippocampal cells were determined using Hoechst fluorescent staining of cell nuclei.

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

## Results

Results of the anti-A $\beta$  1–42 ELISA analysis of conditioned medium (CM) samples from hippocampal cultures exposed for 3 days to a toxic dose (100 nM) of Tat or gp120 revealed a significant increase in the A $\beta$  1–42 production (Figure 1). At the age of 14 days in vitro (DIV), approximately 20–25% of neurons in hippocampal cultures express amyloid protein precursor (APP) isoforms and 1.5–3.0 nM of extracellular A $\beta$  1–42 immunoreactivity can be detected in the cell culture medium. According to the anti-A $\beta$  1–42 ELISA measurements, CM samples from cultures treated with Tat; gp120; or vehicle contained subsequently  $28.2 \pm 1.6$  nM;  $18.4 \pm 5.2$  nM; or  $2.4 \pm 1.4$  nM of the immunoreactive A $\beta$  1–42.

The analysis of A $\beta$  1–42 immunoreactivity in hippocampal cell cultures treated with 50 nM dose of Tat 1–86 B for different periods of time (typical protocol for the Tat cytotoxicity analysis described in [3]) revealed that the increase of soluble A $\beta$  1–42 in CM from hippocampal cell cultures becomes evident early after the start of the exposure and precedes the cell viability decline (Figure 2A,B). The maximum level of specific A $\beta$  1–42 immunoreactivity in the CM occurred after 24-hour Tat exposure and was  $46.1 \pm 17.1$  nM.

Mutations of single cysteine residues at the position 22 or 31 within the conservative cysteine-rich domain of Tat dramatically decrease the ability of Tat to cause apoptosis [3,17]. Unlike with 100 nM dose of Tat 1–86 B, the 48-hour exposure to the same dose of either HIV-1 B Tat 1–86 with cys22→gly22 substitution (Cys22 Tat B) or the HIV-1 C Tat 1–101 in which the cysteine 31 is mutated, did not induce cytotoxicity and did not increase A $\beta$  1–42 production in hippocampal cell cultures (Figure 3A,B).

Staining with Congo Red is a classical method of the detection of amyloids. Compared to other amyloid-binding dyes, Congo Red exhibits very low non-specific binding and cannot be internalized by the cells probably because of the presence of two hydrophilic sulfonic groups. We used the Congo Red staining to determine the potential ability of Tat or gp120 to promote accumulation of amyloidal aggregates in cultured hippocampal cells.

In young mature (14 DIV) hippocampal cell cultures no more than 1% of cells were Congo Red-positive. Consistent with previous studies carried out using this procedure [27], we observed that the exposure to pre-aggregated A $\beta$  1–42 increased the Congo Red staining of cultured hippocampal cells. Approximately 50% of all cells were positively stained with Congo Red following 24-hour incubation with 25  $\mu$ M of pre-aggregated A $\beta$  1–42. The exposure to 50 nM Tat 1–86 B also caused the increase in Congo Red staining suggesting the accumulation of amyloidal aggregates in hippocampal cells (Figure 4). Increased numbers of Congo Red-positive cells ( $17.2 \pm 3.5\%$ ,  $P < 0.05$ ) were detected in cultures exposed to Tat 1–86 B for 4 hours. After 24 hours of treatment,  $26 \pm 3\%$  of the hippocampal cells were positively stained with Congo Red. In cultures treated with 50 nM Cys22 Tat 1–86 B or Tat 1–101 C, which do not induce A $\beta$  production, the results of Congo Red staining were the same as in vehicle-treated controls (data not shown).

In hippocampal cultures treated for 3 days with 100 nM Tat 1–86 B,  $23.3 \pm 1.6\%$  of the remaining cells were positively stained with Congo Red. Relative numbers of Congo Red-positive cells in cultures exposed to 100 nM gp120 ( $2.0 \pm 1.3\%$ ) were not significantly different from vehicle-treated controls ( $1.4 \pm 1.0\%$ ) (Figure 5).

## Discussion

Several recent studies [10,21] have reported that HIV-1 Tat can increase the level of A $\beta$  1–40 in neuronal cell cultures. Consistently, we present the evidence of the increased secretion of highly amyloidogenic variant of A $\beta$  peptide, A $\beta$  1–42, in hippocampal cell cultures exposed to Tat 1–86 clade B. Our results also show that the biogenesis of A $\beta$  in hippocampal cell cultures may be influenced not only by Tat, but by gp120 as well. Hence, changes in the APP/A $\beta$  metabolism may be a shared molecular event of the neuronal response to injury incited by two different HIV proteins that are thought to contribute to the development of cognitive deficits associated with chronic HIV-1 infection *in vivo*.

The increased A $\beta$  production can be associated with various types of neurotoxic insults [14, 15]. Notably, the stimulation of NMDA receptors, which is a common component of HIV-1 Tat and gp120-mediated toxicity mechanisms [16], is known to increase A $\beta$  release in hippocampal neurons [15]. Therefore, it is not surprising that Tat and gp120 can share the ability to affect A $\beta$  production.

Supportive to the idea that the release of soluble A $\beta$  peptides is linked to the HIV protein-mediated neurotoxicity mechanisms are our experiments demonstrating that the Tat variants with attenuated pro-apoptotic potential were unable to increase the A $\beta$  1–42 secretion. Interestingly, patients infected with the subtype C of HIV-1 virus have been shown by other researchers to have decreased incidence of cognitive deficits [20].

The A $\beta$  derivatives of APP are known to have dualistic effects on neuronal function and viability [12,18]. Non-aggregated A $\beta$  monomers have a role in the support of neuronal repair, but their conversion into oligomers with anti-parallel  $\beta$ -sheet structure leads to neurotoxicity [18,24]. The irreversible, spontaneous insertion of amyloid protein aggregates into neuronal cell membranes impairs synaptic transmission and eventually can cause death of affected neurons. Strong evidence suggests that the first essential elements in the vast majority of neurodegenerative processes are misfolded and aggregated proteins [26]. Most amyloid peptides interact strongly with cell membranes and this interaction is enhanced by conditions which favor  $\beta$ -sheet formation. For the first time our study presents the direct evidence that Tat 1–86 B not only increases A $\beta$  release, but also causes the accumulation of amyloid protein aggregates in hippocampal cells.

Unlike with Tat, gp120-induced A $\beta$  1–42 production was not associated with the increase in numbers of Congo Red-positive cells in hippocampal cultures. This result indicates that the ability to simultaneously increase A $\beta$  secretion and facilitate the accumulation of amyloid aggregates in neuronal membranes may be the unique property of Tat. Inhibitory effects of HIV-1 Tat on proteolytic enzymes involved in degradation of damaged and misfolded proteins [5,10,19,23] as well as Tat interaction with the low density lipoprotein receptor-related protein (LRP) involved in re-uptake and clearance of amyloid peptides [13] may underlie the observed ability of HIV-1 Tat B to promote deposition of cell-bound amyloids in hippocampal cell cultures. The recently obtained *in vivo* evidence of increased deposition of aggregated A $\beta$  in the brain of Tat/PS1/APP transgenic mice [13] is in line with our cell culture findings.

The conditions for culturing of hippocampal cells isolated from the hippocampus of E18 rat fetuses are designed to restrict glial proliferation. Primary fetal hippocampal cell cultures do not contain microglia and include no more than 10–20% (depending on the cell culture age) of astrocytes [8]. Neurotoxic effects of gp120 are largely mediated through interactions with glial cells and it is possible that indirect mechanisms involving microglia/macrophages or macroglia may be essential to facilitate aggregation of extracellular A $\beta$  produced in response to gp120. Hence, despite the lack of ability to increase the number of Congo Red-positive

hippocampal cells *in vitro*, gp120-mediated changes in A $\beta$  production may lead to the formation of neurotoxic amyloidal oligomers *in vivo*.

As indicated by this study, Tat and gp120, which are known to cause hippocampal-dependent memory deficits, demonstrate the common ability to induce production of A $\beta$  1–42 *in vitro*, in hippocampal cell culture. The reported results draw attention to the specific connection between the cytotoxic potential of the HIV-1 protein (Tat) and the increased production of A $\beta$ . Further, new evidence is presented indicating that Tat B may possess a unique property to directly stimulate both A $\beta$  biogenesis and accumulation of cell-bound  $\beta$ -amyloids. Via the ability to trigger innate immunity defense mechanisms, amyloid- $\beta$  oligomers formed with the participation of neurotoxic HIV-1 proteins may contribute to the setup of a vicious cycle of neuronal apoptosis, inflammation, and immune dysregulation [22] that results in the progressive decline of memory performance in the HIV-infected brain. In a broader sense, our study supports the idea that the HIV-associated cognitive dysfunction may be a variant of so-called "amyloid pore disease" [26] and rationalizes testing of the potential ability of anti-amyloidogenic compounds to attenuate HIV protein-induced neuronal injury.

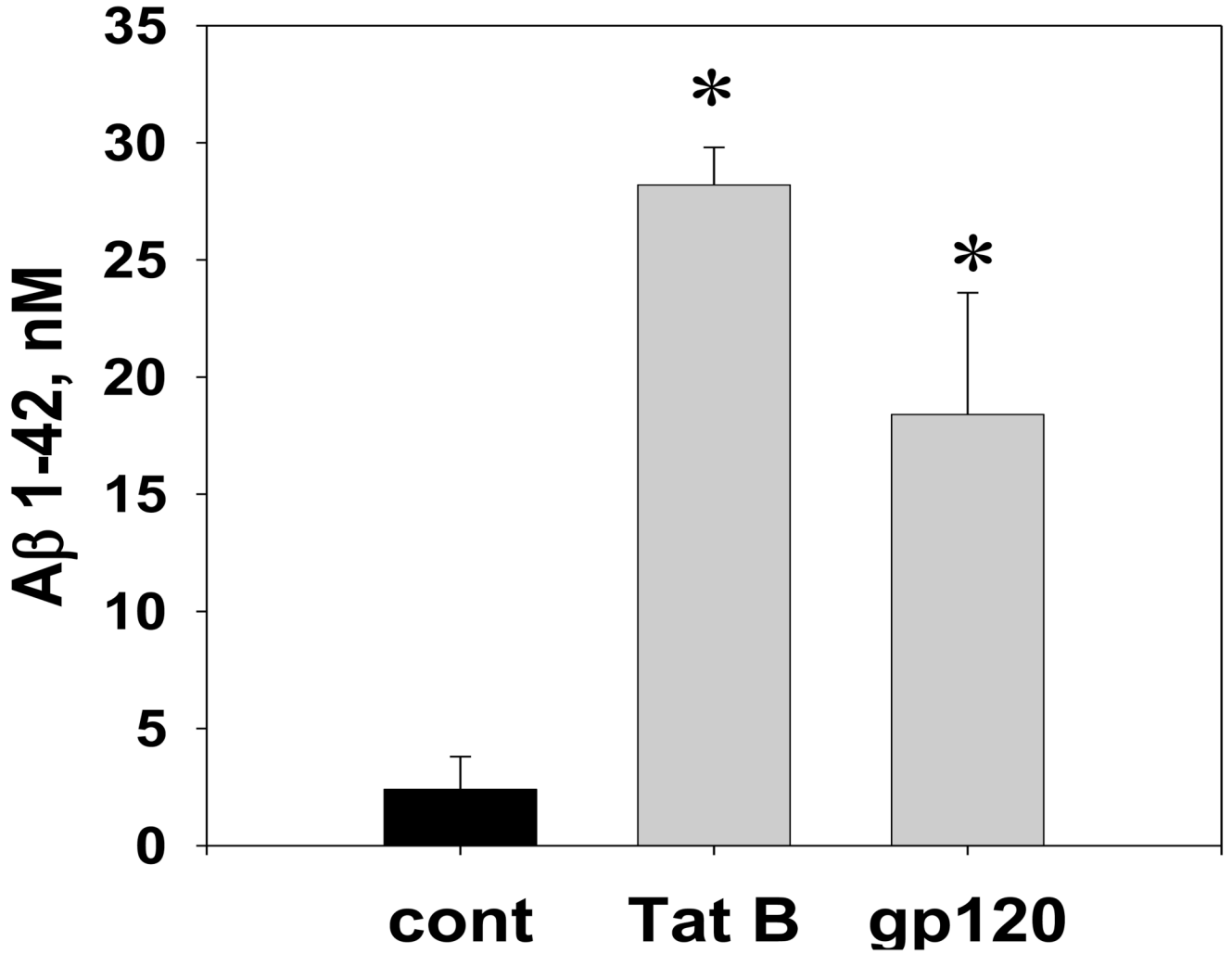
## Acknowledgments

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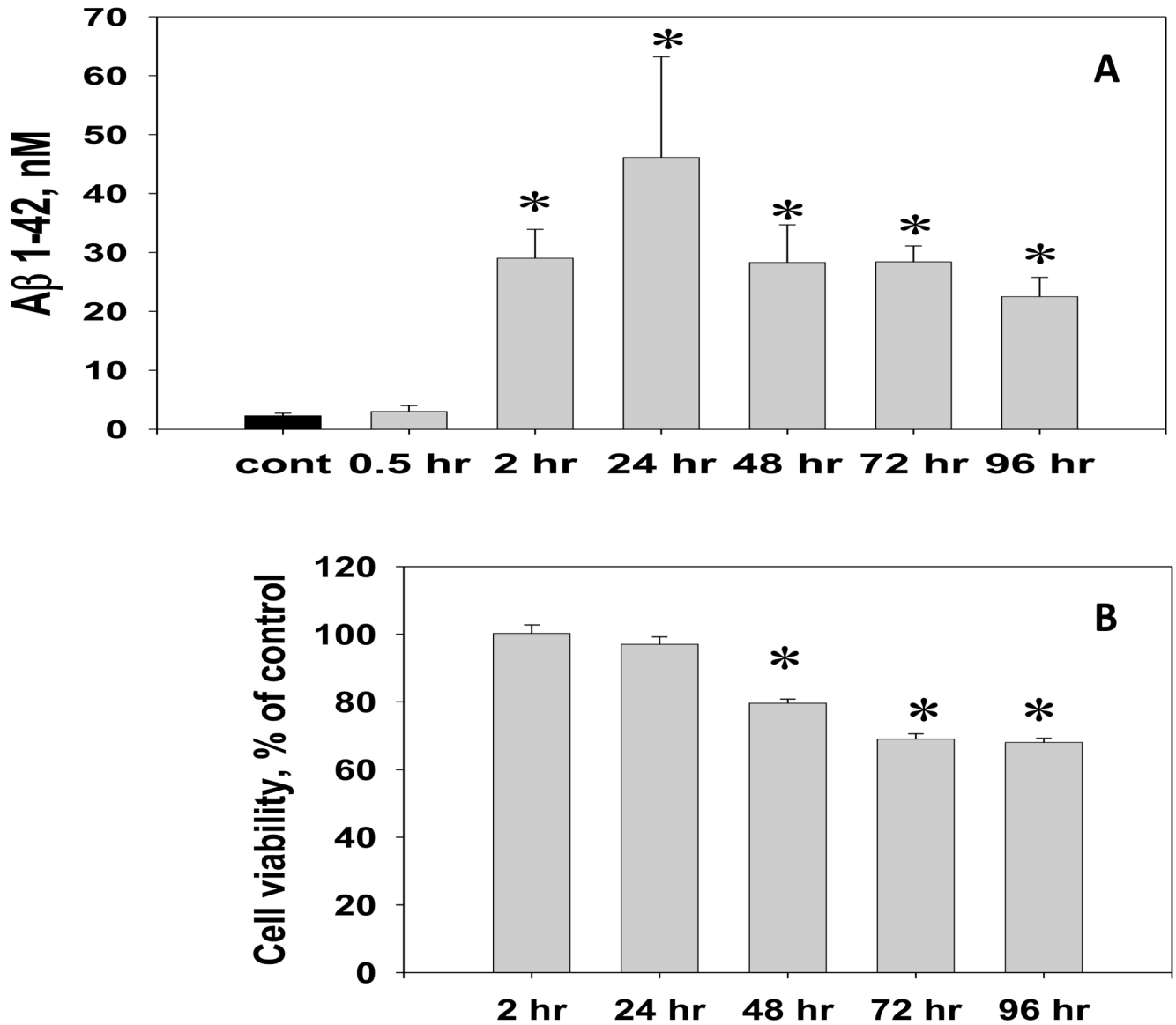
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**Figure 1. The Aβ 1–42 immunoreactivity in the conditioned medium from hippocampal cell cultures exposed to HIV-1 Tat or gp120**

The graph represents results of direct anti-Aβ 1–42 ELISA measurements in the CM samples collected from hippocampal cultures (n=8 per group) treated with either 100 nM Tat 1–86 B, 100 nM gp120, or the equal volume of vehicle. The freshly prepared Aβ 1–42 stock was serially diluted with cell culture growth medium to obtain ELISA calibration curves. Data presented as mean values ± SEM. \*- marks significant (P<0.05) differences in extracellular Aβ 1–42 immunoreactivity.

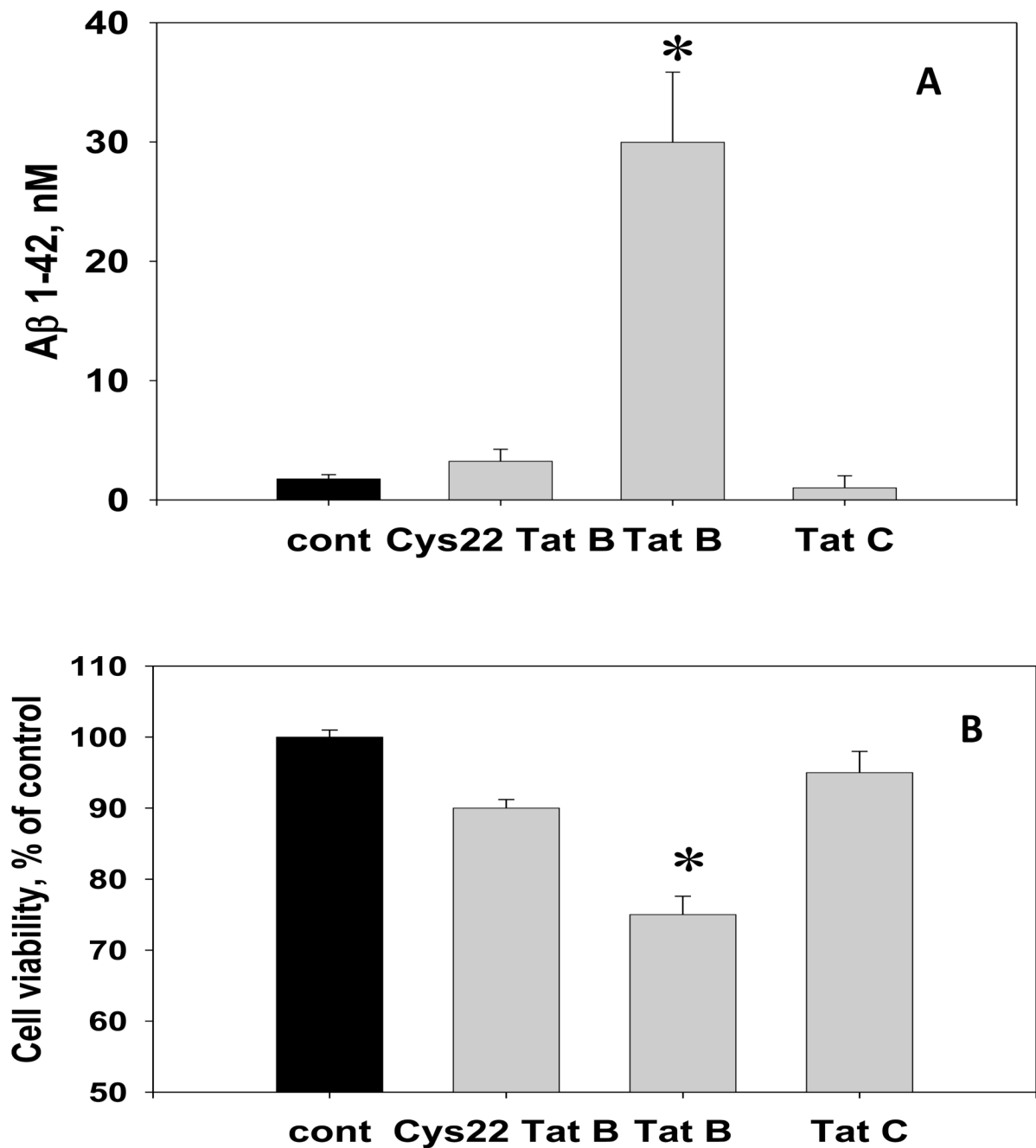


**Figure 2. The temporal relationship between the Aβ 1–42 release and cell viability changes in hippocampal cell cultures exposed to a toxic dose of Tat 1–86 B**

**A.** The Aβ 1–42 immunoreactivity was measured in CM samples collected from hippocampal cultures during the continuous exposure (0.5 – 96 hours) to 50 nM Tat 1–86 or vehicle (n=5 per group). Data are presented as mean values ± SEM. \*- marks significant (P<0.05) differences in extracellular Aβ 1–42 immunoreactivity between Tat-treated and vehicle-treated cell cultures.

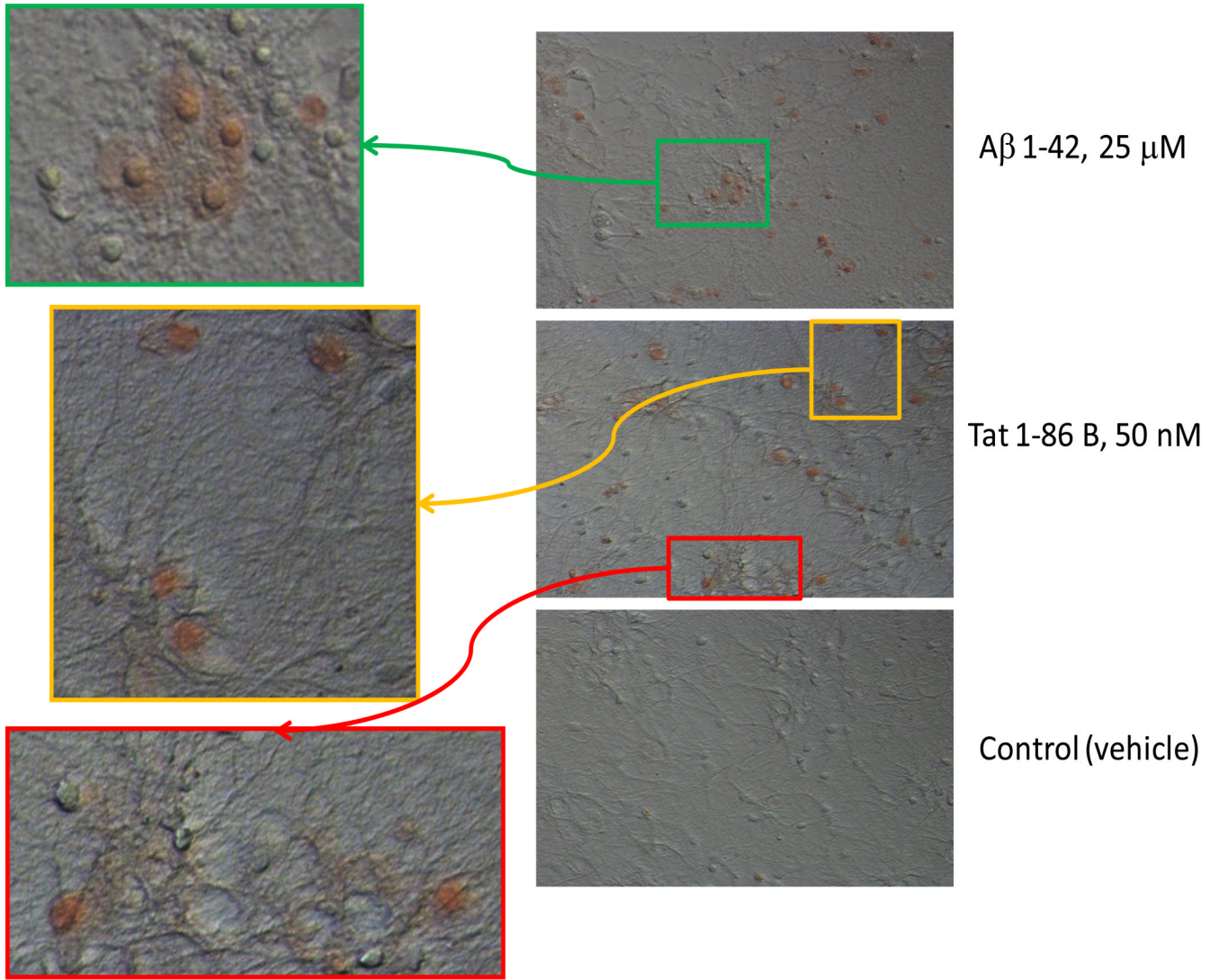
**B.** Cell viability in hippocampal cell cultures (treated side-by-side, n=8 per group) was determined using Live/Dead assay. Data are presented as mean % of control values ± SEM. \*-marks incubation time points for significant (P<0.05) differences in Live/Dead ratios between cultures treated with 50 nM Tat 1–86 and vehicle-treated controls have been observed.





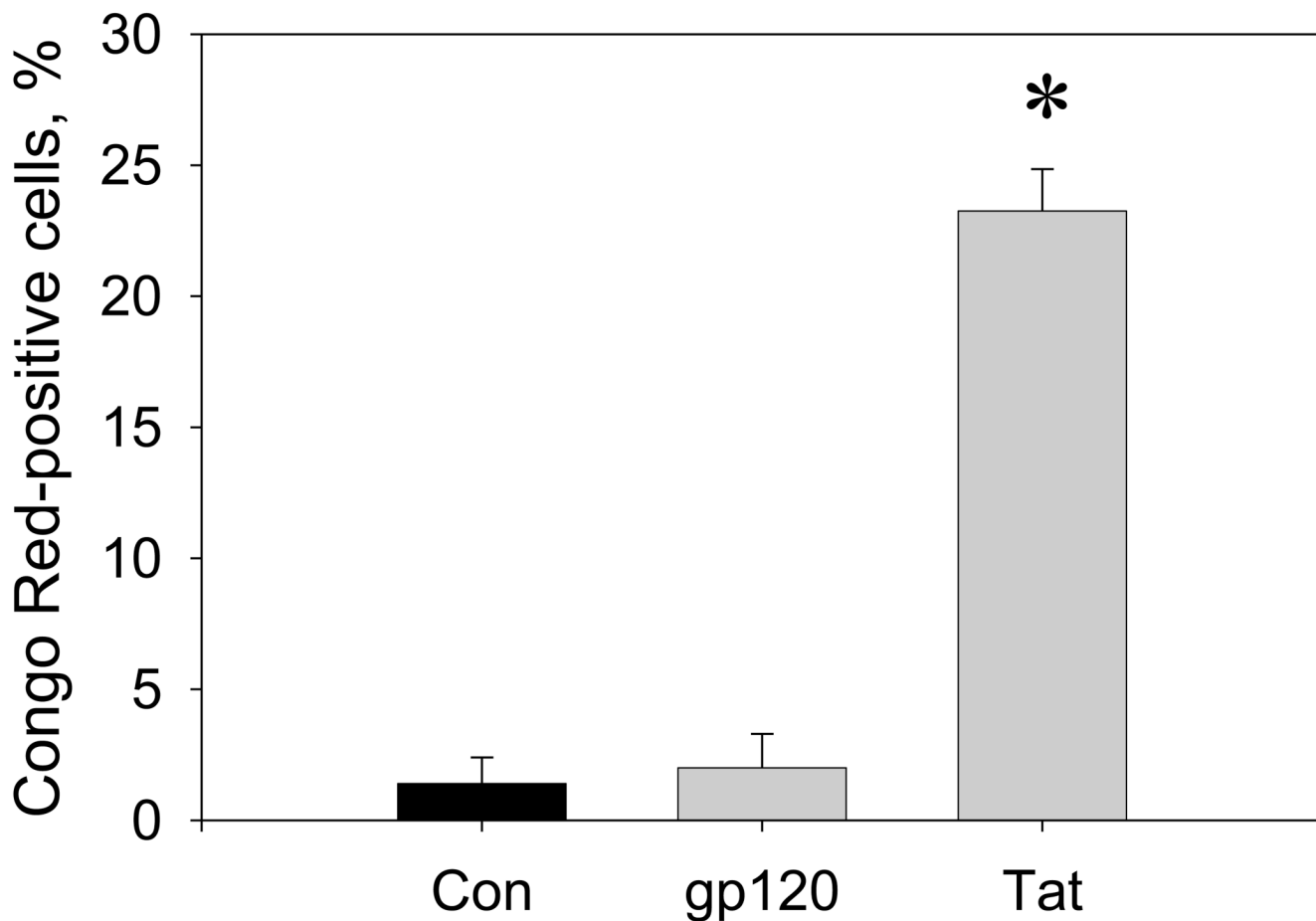
**Figure 3. The effect of non-neurotoxic variants of HIV-1 Tat (Cys22 Tat 1–86 B and Tat 1–101 C) on the Aβ 1–42 production in hippocampal cell cultures**

The graph represents results of direct anti-Aβ 1–42 ELISA measurements in the CM samples collected from individual cultures (n=8 per group) treated with 100 nM Tat 1–86 B, Cys22 Tat 1–86 B, Tat 1–101 C, or the equal volume of vehicle for 72 hours. Data are presented as mean values ± SEM. Cell viability in hippocampal cell cultures was determined using Live/Dead assay. \*- marks significant (P<0.05) differences in hippocampal cell viability or extracellular Aβ 1–42 immunoreactivity.



**Figure 4. The detection of cell-bound  $\beta$ -amyloid aggregates by the Congo Red staining in living hippocampal cells exposed to  $A\beta$  1-42 or HIV-1 Tat 1-86 B**

Representative images show the Congo Red binding to hippocampal cell cultures exposed to a toxic dose of  $A\beta$  1-42 (40-hour exposure), Tat 1-86 B (24-hour exposure), or vehicle. Results of the Congo Red staining in cultures treated with 100 nM gp120 for 72 hours, 100 nM Cys22 Tat or Tat 1-101 C for 24 hours were not different from the vehicle-treated controls and are not shown in the Figure. Colored boxes mark areas of Congo Red/DIC images, which are shown as new magnified images with greater resolution.



**Figure 5. Changes in numbers of Congo Red-positive cells in hippocampal cell cultures exposed to HIV-1 Tat or gp120**

The graph represents relative numbers (% of total cells) of Congo Red-positive cells in hippocampal cell cultures after 3 days of treatment with vehicle (control), gp120 (100 nM), or Tat 1–86 B (100 nM). For each individual cell culture in a group (n=4), Congo Red-positive hippocampal cells were counted in 4 random fields of vision. For each field of vision (0.5×0.37 mm) results were normalized to the subsequent total cell number determined using Hoechst staining of cell nuclei (typically 70–95 cells per field). Data are presented as mean values ± SEM. \*- marks significant (P<0.05) differences in relative numbers of Congo Red-positive cells.