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Delivery of macromolecules into live cells by simple co-incubation with a peptide

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The delivery of biosensors and bioactive proteins into live cells can enable the manipulation or probing of intracellular processes in powerful ways. To date, targeting large and/or hydrophilic molecules across the plasma membrane of large numbers of cells rapidly and efficiently remains a challenge. An attractive approach to solving this problem consists of utilizing cell-penetrating peptides (CPPs) such as the prototypical HIV TAT peptide (GRKKRRQRRR) as delivery systems.[1] When conjugated to macromolecules such as peptides, proteins and quantum dots, CPPs have the ability to carry their cargo into cells, both *in vitro* and *in vivo*. [2,3] However, CPPs have been demonstrated to alter the intracellular localization of the macromolecule to which they are conjugated once cytosolic delivery is achieved. For instance, TAT and other arginine and lysine-rich CPPs induce accumulation of their conjugated cargos at the nucleoli of cells (Supplementary Figure S1). [4–6] This results in an undesirable fluorescence signal from improperly targeted biosensors in imaging applications. In addition, it raises the concern that the function of delivered probes or the activity of bioactive agents might be deeply compromised by the delivery peptide. A solution to this problem is to establish a CPP to cargo linkage that can be disrupted once the macromolecule reaches the cytosolic space so as to generate an unmodified species once delivery is achieved. Examples of such strategies include the use of disulfide bonds that can be reduced inside cells or of peptides that bind non-covalently to macromolecules to form carrier peptide/cargo particles.[7–11] This latter strategy has in particular been applied to the delivery of nucleic acid sequences with great success.[12–16] Cationic CPPs can for instance interact electrostatically with negatively charged DNA or RNA sequences to form CPP/nucleic acids complexes that can cross the plasma membrane of cells. Similarly, hydrophobic CPPs that can bind non-covalently to hydrophobic regions of proteins have been used to successfully deliver these macromolecules to live cells.[7,17] These approaches can however be problematic since the efficiencies of CPP/protein complex formation and delivery are dependent on the identity of the protein cargo. Simple and general delivery methods that would address the aforementioned problems are therefore still required.

In this report, we test the hypothesis that optimized CPPs might deliver macromolecules to the cytosol of live cells by simple co-incubation and without the requirement for any type of conjugation, whether covalent or non-covalent. This hypothesis is based on the observation that the binding of TAT and related arginine-rich CPPs to the cell surface promotes increased levels of endocytosis.[18–20] We therefore reasoned that a CPP might induce a

cell to “drink-up” macromolecules present in its surrounding media, and that CPPs and macromolecules might accumulate together inside endocytic organelles. A CPP capable of disrupting these endocytic organelles might then induce the release of the macromolecules into the cytosolic space. Importantly, the macromolecules would not have to be modified with the CPP and any deleterious effect that the CPP could have on the macromolecule’s function or localization once the cargo is delivered into the cell would be avoided. Although this co-incubation protocol might not be suitable for the delivery of molecules in therapeutic applications, it would be simpler and less invasive than other multistep delivery processes (e.g. osmotic lysis of pinocytic vesicles, plasma membrane permeabilization, scrape loading, or microinjection) and therefore represents a step towards making the delivery of macromolecules to cultured cells a routine procedure for imaging or cell biology applications.[21–23]

In order to identify a CPP capable of delivering macromolecules efficiently in trans, the CPPs TAT, and the HA2-TAT chimeras E3-TAT (GLFGA IAGFI ENGWE GLIEG WYGGR KKRRQ RRR), and E5-TAT (GLFEA IAEFI ENGWE GLIEG WYGGR KKRRQ RRR) were characterized in cell-based assays. HA2 is an amphiphilic peptide derived from the hemagglutinin protein of the *Influenza* virus that can disrupt lipid bilayers in a pH-dependent manner.[24–26] HA2 has been shown to complement TAT by disrupting the membrane of endosomes upon acidification of these organelles and has been used to achieve greater cytosolic release of TAT-conjugated cargos.[3,18,27–30] We chose to fuse TAT to E3 and E5, two HA2 variants with increasing membrane disruption activities (e.g. E5 is more active than E3 in liposome leakage assays) so as to generate CPPs with potentially different endosomolytic activities.[31,32] TAT, E3-TAT, and E5-TAT were prepared by solid-phase peptide synthesis. To identify an effective concentration of CPP that could be used for the delivery of macromolecules without inducing cell-death, the cytotoxicity of these peptides toward HeLa cells was first examined using the MTT [=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay (Figure 1) and by observation of the cell morphology by phase-contrast imaging. Cells were exposed to the CPPs at different concentrations for 1h, washed and incubated in L-15 media from 3 to 24 hours before being assayed for viability. TAT was essentially non-cytotoxic under the conditions tested (1 to 20 μ M) according to the MTT assay. However, above 10 μ M, the peptide appeared to affect the morphology of a large population of cells. E3-TAT and E5-TAT did not induce a significant decrease in cell viability at concentrations below 2.5 μ M. In contrast to TAT, the cytotoxicity of the peptides were, however, more acute at 5 μ M and above. Overall, to minimize the physiological effects that the peptide might have on cells, concentrations of 10 μ M of TAT and 2 μ M of E3-TAT and E5-TAT were therefore set as upper limits for testing the delivery of fluorescent cargos to live cells.

To characterize the CPP-mediated delivery of macromolecules in live cells, fluorescent dextrans and proteins were chosen as model cargos, including 10 and 70 kDa anionic Dextran-fluorescein, 10 and 70 kDa neutral Dextran-tetramethylrhodamine, 70 kDa neutral Dextran-Texas Red, the green fluorescent protein EGFP (27kDa, pI = 5.7, –7 charges at pH 7.2), and the red fluorescent proteins mCherry (28kDa, pI = 6.0, –4 charges at pH 7.2), MBP-mCherry (71kDa, pI = 5.5, –10 charges at pH 7) and tdTomato (55kDa, pI = 6.3, –6 charges at pH 7.2).[33] These molecules have different sizes and overall charges as well as different chemical structures that might affect uptake and localization within the cells. Genetically encoded fluorescent proteins were tested because these proteins lose their fluorescence if unfolded or proteolytically cleaved.[10] Detection of their fluorescent emission inside a cell therefore serves as an indication that the proteins are not degraded. On the other hand, their fluorescent signals might disappear with time if the proteins remain trapped inside degradative organelles such as lysosomes. Dextrans are typically resistant to intracellular degradation, including degradation within the endolysosomal pathway.[34] As a

result, Dextran-tetramethylrhodamine and Dextran-Texas Red provide useful markers to measure and compare the endocytic or cytosolic distribution of fluorescent material internalized into a cell (fluorescein is pH sensitive and its fluorescence is partially quenched in the acidic lumen of endocytic organelles but tetramethylrhodamine and Texas Red are relatively insensitive to pH).[10] When co-incubated with the different fluorescent cargos, TAT, E3-TAT, and E5-TAT did not affect the fluorescence emission or anisotropy of the various fluorescent molecules tested, indicating that the peptides do not bind to the cargos at detectable levels (data not shown). Native gel electrophoresis analysis of the peptide/protein mixtures further confirmed that, while TAT might bind to negatively charged proteins to generate low concentrations of peptide/protein complexes, E5-TAT does not appear to interact with the fluorescent proteins tested at detectable levels (Supplemental information, Figures S2 and S3).

In a typical cell-based delivery assay, the CPPs (2 μ M) were incubated with two fluorescent cargos, one larger and one smaller than the nuclear diffusion threshold (~50 kDa).[35] Cell viability during these assays was determined by SYTOX® blue staining, and by observation of active transport of intracellular endocytic organelles with time-lapse imaging (Supplemental information, Figure S4). HeLa (human cervical adenocarcinoma), COS-7 (SV40 transformed African green monkey kidney fibroblast-like cell line), and COLO 316 (human ovarian carcinoma) cells were incubated with the fluorescent macromolecules (10 μ M for the proteins, 2.5 mg/mL for the dextrans, or approximately 250 μ M and 36 μ M for 10 kDa and 70 kDa dextrans, respectively), in combination with a CPP (2 μ M) for 1 hour. After washing the cells, internalization of the fluorescent material was imaged by live-cell fluorescence microscopy (Figure 2).

Under the conditions tested, cells incubated with just the macromolecules and no CPP displayed an intracellular punctate distribution of fluorescent markers of relative low fluorescence intensity. This distribution is consistent with the fluorescent markers being internalized by fluid-phase endocytosis at basal level and remaining trapped inside endocytic organelles. In contrast, cells incubated with the CPPs partitioned into two distinct populations: cells with a punctate labeling and cells with diffusely distributed fluorescent cargos (these two populations are illustrated by the left-side image of Figure 2 a, please note that 100% of the cells contained the fluorescent macromolecules but that fluorescence is either localized inside endocytic organelles or distributed throughout the cell). Nuclear exclusion of the large cargo served as a demonstration that the fluorescent signal observed was indeed intracellular rather than out-of-focus fluorescence of the cargos bound to the plasma membrane (right-side image in Figure 2 a, the nucleus is labeled with the 10 kDa Dextran-fluorescein but not with the larger 70 kDa Dextran-Texas Red). When TAT was used as delivery peptide, the population of the cells containing a diffuse distribution of the fluorescent cargos was less than 1% of the total cells observed (e.i. more than 99% of cells contain the fluorescent macromolecules in endocytic vesicles). No significant differences were observed regardless of the concentration tested (1 to 10 μ M). In contrast, the number of cells with a diffuse cytosolic distribution was $8\pm 2\%$ and $15\pm 3\%$ on average when E3-TAT and E5-TAT were used, respectively (2 μ M, Figure 3 a, the results represented are for mCherry and are comparable to the other macromolecules tested, see Supplementary Figure S5). Based on the measurement of the fluorescence intensities, the cytosolic/nuclear signals of cells with a diffuse distribution were 50-fold above that of cells with a punctate distribution. Similar results were obtained in HeLa, COS-7 or COLO 316 cells (Figure 2 b).

The total fluorescence intensity of cells incubated with the macromolecules and TAT, E3-TAT or E5-TAT were three to five fold higher than that of cells incubated without delivery peptides, indicating that increased levels of cellular uptake had been induced by the peptides (Figures 3 b and S5, the fluorescence intensities of whole cells are measured to estimate the

average amount of fluorescent material internalized inside cells). Internalization of the fluorescent cargos into endocytic organelles was inhibited at 4°C, indicating that uptake is an energy-dependent process. Internalization was also inhibited by heparin, a molecule known to inhibit the binding of TAT to heparan sulfate proteoglycans or other negatively charged molecules present on the cell surface (Figure 3 a).[36] This is consistent with previous mechanistic studies of TAT-mediated uptake and suggests that the TAT moiety in E3-TAT or E5-TAT is in large part responsible for the elevated endocytic uptake of the cargos.[18] The vacuolar H⁺-ATPase inhibitor bafilomycin was used to inhibit endosomal acidification prior to incubation with E3-TAT or E5-TAT and the fluorescent cargos.[28] The number of cells containing a diffuse distribution of fluorescent cargos was greatly reduced by this treatment from 8% or 15% to less than 0.5% (Figure 3, left-side panel). The amount of material internalized was however unchanged based on the fluorescence intensities of cells containing a punctate distribution of cargos (Figure 3, right-side panel). This indicates that the presence of cells with diffusely distributed fluorescent cargos is dependent on the acidification of the lumen of endocytic organelles and that E3-TAT and E5-TAT mediate endosomal release through their pH-dependent membrane disruption activity.

To determine whether E5-TAT could mediate the delivery of a biologically active cargo, the delivery of the pro-apoptotic domain peptide KLAKLAKKLAKLAK-NH₂ (PAD) into HeLa cells was evaluated.[37,38] If present inside cells, PAD has been shown to induce apoptosis by triggering mitochondrial permeabilization and depolarization.[38,39] However, PAD cannot cross the plasma membrane by itself and shows low cytotoxicity in *in vitro* assays when no delivery agent is used.[38] In our assay, PAD was therefore co-incubated with TAT or E5-TAT and cell viability, evaluated with the MTT assay, was used as an indirect measure of the efficiency with which PAD reaches the cytosolic space and the mitochondria. As shown in Figure 4 a, co-incubation of TAT (5 μM) with PAD (20 μM) did not cause more cell-death than treatment with PAD (20 μM) alone, suggesting that TAT is not able to deliver PAD efficiently. In contrast, addition of increasing concentrations of E5-TAT to PAD induced an increase in cell death. In order to confirm that the cytotoxicity levels observed were due to an increase in delivery of PAD into cells by E5-TAT as opposed to non-delivery related effects of the combined peptides, this assay was also performed with cells treated with amiloride. Amiloride is an inhibitor of macropinocytosis, a form of endocytosis known to be important for the uptake of TAT-cargo conjugates into cells.[18,40,41] We therefore reasoned that macropinocytosis could be involved in the uptake of E5-TAT and that inhibition of macropinocytosis might fully or partially block E5-TAT mediated delivery of PAD. In agreement with this idea, cell death was significantly reduced upon amiloride treatment. These results therefore suggest that inhibition of macropinocytosis reduces the delivery of PAD into cells and that the cytotoxicity effects observed are in great part due to the E5-TAT-mediated delivery of a bioactive PAD into the cells (the cytotoxicity observed in the presence of amiloride could be caused by incomplete inhibition of the transport of PAD; it is also consistent with the cytotoxicity observed for E5-TAT alone as shown in Figure 1). These results were further confirmed by monitoring by fluorescence microscopy the delivery of a PAD peptide labeled with the red fluorophore carboxytetramethylrhodamine, TMR-PAD. Cells incubated with both TMR-PAD and E5-TAT displayed a cytoplasmic red fluorescence emission consistent with delivery of PAD in their cytoplasm (Figure 4 b) while cells treated with TMR-PAD alone did not (not shown). Cells containing the red fluorescent PAD became permeable to the SYTOX® blue DNA stain approximately 30 min after incubation with the peptides was completed. The cells also displayed marked changes in their morphology consistent with apoptosis, including membrane blebbing, contraction, and nuclear fragmentation (not shown). Importantly, cells that were not red fluorescent did not become stained by SYTOX® blue. In addition, the control peptide TMR-K9 was also delivered into the cytosol of cells by E5-TAT co-

incubation. These cells did not become apoptotic or permeable to SYTOX Blue (not shown, see Figure S4 for similar experiments performed with mCherry). These results indicate that delivery alone is not causing cell death but that the cytotoxicity observed is a result of the activity of PAD. Because cells containing TMR-PAD were dying during the experiment, an exact quantification of the delivery efficiency could not be determined by microscopy. However, based on the MTT assay, it appears that E5-TAT was able to deliver a lethal dose of active PAD to 15–25% of cells (Figure 4 a, as established by the difference in cell viability observed between the cells with or without amiloride).

Overall, these results suggest that TAT performs poorly for the delivery of macromolecules in trans. Addition of endosome disrupting peptides E3 or E5 however enhances the activity of the peptide and a significant fraction of cells containing macromolecules in the cytosolic and nuclear spaces can then be observed. E3/5-TAT delivery is mediated by endocytosis and endosomal release, rather than by direct translocation across the plasma membrane (Figure 5). First, the TAT moiety binds to the cell surface and induces an increase in endocytic uptake. Macromolecular cargos present in the media surrounding the cell are then internalized along with the delivery peptide into endosomes. Upon progressive acidification of the lumen of endosomes, the E3/E5 moiety disrupts the endosomal membrane and the cargos escape into the cytosol. E5 is known to induce membrane leakage more actively than E3, and consistent with this observation, E5-TAT appears to deliver fluorescent cargos into cells more efficiently than E3-TAT.

Multiple macromolecules with very different size, charge, hydrophobicity, and chemical structures could be delivered simultaneously to the cell when co-incubated with E5-TAT. This, along with the fact that no binding interactions between E5-TAT and the macromolecules tested could be detected in *in vitro* assays (fluorescence assays and native gel electrophoresis, Figure S3), suggests that binding of E5-TAT to a macromolecule is not required to achieve delivery. As a result, no chemical or genetic modification of the cargo is required, thereby allowing for a simple “mix and incubate” protocol to be performed. Moreover, the function of the delivered macromolecule is not altered by the delivery peptide once delivery is achieved. Preliminary delivery experiments with HA2-TAT-mCherry, a macromolecule where the delivery peptide HA2-TAT is fused to the mCherry cargo, established that the membrane affinity of the HA2-TAT moiety promotes binding of mCherry to intracellular membranes and that, as a result, the protein remains tethered to endosomes and is able to escape into the cytosolic space (Supplemental Information, Figure S7). This further emphasizes that, at least for *in vitro* assays, a “non-conjugation” approach might be preferable. It should be noted however that the E5-TAT delivery method presented herein could involve the non-covalent binding of the peptide to its cargo depending on the nature of the macromolecule used for delivery. Although no binding was detected for the various cargos used in this report (Figure S3), it is conceivable that highly negatively charged species (proteins with low isoelectric point, RNA, DNA) might interact with the arginine-rich TAT through multiple electrostatic interactions (Supplemental information, Figures S2 and S3). However, it is also possible that such non-covalent interactions might induce a more efficient delivery. Delivery by co-incubation with E5-TAT might therefore serve as a general approach, whether or not non-covalent interactions are involved between peptide and cargo.

In terms of applications, the activity of E5-TAT was sufficient to deliver imaging agents at a level that was well above the detection limit of a standard fluorescence microscopy set-up. A limitation however is the relatively low frequency of cells containing a clear diffuse distribution of the fluorescent agents. Although this might be suitable for certain assays (e.g. 15% of several thousand cells is many more cells than what one would obtain via microinjection), it is inconvenient as it renders the imaging of multiple cells in the field of

observation unlikely at high magnification. Future improvements should therefore be directed at increasing this aspect of the peptide's activity. Interestingly, the E5-TAT delivery appeared to be a all-or-none process where cells are distributed between two distinct populations: either the cargo reaches the cytosol with little appearing to be left inside endosomes, or it almost completely remains trapped in numerous endocytic organelles. Understanding this phenomena will likely involve characterizing further how the peptide functions. In particular, it is possible that E5-TAT and the internalized macromolecules partition into different endocytic organelles during or soon after uptake, thereby reducing the overall effectiveness of the delivery (Supplemental Information, Figure S6). In principle, other TAT-like molecules that could trigger different endocytic mechanisms of uptake combined to HA2-like moieties with greater endosomolytic activities might solve this problem and provide more efficient and general delivery systems.

Experimental Section

Cytotoxicity Assay

HeLa cells were seeded on 96-well tissue culture plates at 1.0×10^4 cells/well. After incubation in a humidified atmosphere containing 5% CO₂ for 24 h at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, the cells were washed with Leibovitz's L-15 medium and treated with TAT, E3-TAT, and E5-TAT in L-15. After 1 h incubation, the cells were washed with fresh L-15 medium and incubated at 37°C for 3 hours. The number of live cells present in each well was then determined using the Vybrant MTT Cell Proliferation Assay Kit (Molecular probes, Carlsbad, CA). Alternatively, SYTOX® Blue, a nucleic acid fluorescent stain that only penetrates cells with compromised plasma membranes, was used to measure cell-viability by fluorescence microscopy imaging.

Live Cell Assay for the Transduction of Fluorescent Imaging agents

HeLa, COS-7 and COLO 316 cells were seeded on 8-well chamber glass slide at 3.0×10^4 cells/well in Dulbecco's modified Eagle's medium supplemented with 10% FBS and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂. Cells were then incubated with L-15 medium (no FBS added) and placed on an inverted epifluorescence microscope (Model IX81, Olympus, Center Valley, PA) equipped with a heating stage maintained at 37°C. The microscope is configured with a spinning disk unit to perform both confocal and wide-field fluorescence microscopy. Images were collected using a Rolera-MGI Plus back-illuminated EMCCD camera (Qimaging, Surrey, BC, Canada). Images were acquired using phase contrast and three standard fluorescence filter sets: CFP (Ex = 436 ± 20 nm / Em = 480 ± 40 nm), Texas Red (Ex = 560 ± 40 nm / Em = 630 ± 75 nm), and FITC (Ex = 482 ± 35 nm / Em = 536 ± 40 nm). Cells were co-treated with of TAT or E3/E5-TAT (2 µM) in L-15 medium containing fluorescent imaging agents at 10 µM for the fluorescent proteins EGFP, mCherry, and tdTomato (obtained as previously described) and 2.5 mg/mL for the dextrans 10 and 70 kDa anionic Dextran-fluorescein, 10 and 70 kDa neutral Dextran-tetramethylrhodamine, and 70 kDa neutral Dextran-Texas Red.[10] After incubation at 4 or 37°C for 15 or 60 minutes, the cells were washed with PBS 5 times and the medium was replaced with fresh L-15. The integrity of the plasma membrane of the cells was determined by addition of the cell-impermeable DNA stain SYTOX® Blue. Cells with a blue fluorescent nucleus, detected with the CFP filter set, were considered compromised or dead. The cells that were not stained by SYTOX® Blue were further confirmed to be alive by detection of active transport of intracellular endocytic vesicles. To inhibit cell surface binding of the delivery peptides, heparin sodium salt (1 mg/mL) was added during the co-incubation step. To inhibit acidification of the endolysosomal organelles, cells were pretreated with bafilomycin (200 nM) for 20 min. Cells were then co-incubated with the fluorescent macromolecules and the delivery peptides in L-15 supplemented with

bafilomycin (200 nM). The fluorescence intensities within cells were measured using the SlideBook 4.2 software (Olympus, Center Valley, PA). The mean intensity within cells imaged with the FITC or Texas Red filters was measured as the total intensity of the cell divided by its area. The same protocol was used for cells with either punctate or diffuse distributions. Mean fluorescence intensities were measured over a population of 3000 cells and experiments were reproduced 3 times.

Live Cell Assay for the delivery of PAD

Cells grown on 96-well plates were treated with PAD (20 μ M) and TAT (5 μ M) or E5-TAT (3, 5, or 7 μ M) in L-15. For the inhibition of macropinocytosis, cells were first pretreated with 50 μ M of amiloride (Sigma, MO) for 30 minutes, and then with the peptides while keeping amiloride present. After 1 h incubation, the cells were washed with fresh L-15 medium again and incubated at 37°C for additional 3 hours. The number of live cells present in each well was then determined using the Vybrant MTT Cell Proliferation Assay Kit (Molecular probes, Carlsbad, CA). After 1 h incubation the cells were washed with L-15. SYTOX® Blue (5 μ M) was added and cells were incubated for 15 min at 37°C. Cells were then placed on the microscope and images were acquired in the blue fluorescence channel for detection of SYTOX® Blue and red fluorescence channel for detection of TMR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Viability of HeLa cells incubated with TAT, E3-TAT, or E5-TAT. Cells were treated with the peptides for 1 hour, washed, and incubated in fresh media for an additional 3 hours. Viability is measured as a function of peptide concentration using the MTT assay.

Figure 2.

Live-cell imaging of cells incubated with E5-TAT and green and red fluorescent macromolecules. **(a)** HeLa cells after 1h incubation with E5-TAT and a green and a red fluorescent dextran display a diffuse and punctate distribution of the fluorescent macromolecules. The fluorescence images acquired in the green and red channels are pseudo-colored and overlaid. Co-localization of the green and red fluorescence signals results in a yellow color in the overlay image. The perimeter of the cells is highlighted in the left-hand side image while the cytosolic and nuclear spaces are indicated by white arrows in the right-hand side image. The 10 kDa green dextran diffuses into the nucleus while the 70 kDa red dextran does not and the nucleus appears green. **(b)** Delivery of the protein mCherry and of 70 kDa Dextran-fluorescein in different cell lines. The protein diffuses into the nucleus while the 70 kDa green dextran does not and the nucleus appears red.

Figure 3. Effects of co-incubation conditions and inhibitors on the delivery of the protein mCherry to HeLa cells. Similar Results were obtained for 70 kDa Dextran Texas-Red.

Figure 4.

Delivery of the pro-apoptotic peptide domain PAD into live cells by co-incubation with E5-TAT. **(a)** PAD (20 μ M) and TAT or E5-TAT (3 to 7 μ M) were added to the cell culture media for 1 hour. Delivery of the bioactive PAD was assessed by measuring the level of cell death caused by this incubation. Addition of E5-TAT to PAD results in an increase in cell death while addition of TAT does not. Inhibition of macropinocytosis by treatment of the cells with amiloride reduces cell death. These results therefore suggest that inhibition of macropinocytosis reduces the delivery of PAD into cells and that the cytotoxicity effects observed are in great part due to the E5-TAT-mediated delivery of a bioactive PAD into the cells. **(b)** Delivery of the red fluorescent TMR-PAD by E5-TAT detected by microscopy. The phase contrast and fluorescence images acquired in the blue (SYTOX® Blue detection) and red channels (TMR-PAD detection) are pseudo-colored and overlaid. Cells showing a cytosolic distribution of TMR-PAD become permeable to SYTOX® Blue over time (blue nuclei shown) and apoptotic. This indicates that delivery of PAD precedes cell death and is consistent with the idea that PAD causes apoptosis. Please note that the blue areas in the first image are due to the nuclear debris of cells that have died during the incubation with the peptides and that are stained by SYTOX® Blue.

Figure 5.

Proposed model for the delivery of macromolecules mediated by co-incubation with E5-TAT. Delivery occurs in a two-step process: endocytosis followed by endosomal release. TAT is primarily responsible for the internalization of molecules present in the incubation media by endocytosis while E5 induces escape of the content of endocytic organelles into the cytosolic space. The represented stoichiometry of E5-TAT versus macromolecules is arbitrary.