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TITLE PAGE

Use of penetrating peptides interacting with PP1/PP2A proteins as a general approach for a drug phosphatase technology.

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PP1/PP2A are targets for rational drug design

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d Abbreviations

FITC= Fluorescein isothiocyanate

PP2A1= trimeric ABαC PP2A holoenzyme

CPP= cell penetrating peptide

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ABSTRACT

Type 1 (PP1) and type 2A (PP2A) represent two major families of serine/threonine protein phosphatases that have been implicated in the regulation of many cellular processes including cell growth and apoptosis in mammalian cells. PP1 and PP2A proteins are composed of oligomeric complexes comprising a catalytic structure (PP1c or PP2AC) containing the enzymatic activity and at least one more interacting subunit. The binding of different subunits to a catalytic structure generates a broad variety of holoenzymes. We showed here that Casein Kinase 2α (Ck2α) and SV40 virus small t antigen share a putative common β-strand structure required for PP2A1 trimeric holoenzyme binding. We have also characterized DPT-sh1, a short basic peptide from Ck2α that only interacted *in vitro* with PP2A-A subunit and behaves as a non toxic penetrating shuttle in several cultivated human cell lines and chick embryos. In addition, DPT-sh1 specifically accumulated in human red cells infected with *P. falciparum* malaria parasites. We therefore designed bipartite peptides containing DPT-sh1 and PP1 or PP2A interacting sequences. We found that DPT-5, a DPT-sh1-derived peptide containing a short sequence identified in CD28 antigen, interacts with PP2A-Bα and DPT-7, another DPT-sh1-derived peptide containing a short sequence identified in Bad as a PP1 catalytic consensus docking motif, induce apoptosis in cultivated cell lines. These results clearly indicate that the rational design of PP1/PP2A interacting peptides is a pertinent strategy to deregulate intracellular survival pathways.

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INTRODUCTION

In eukaryotic cells, biological activity of nearly 30% of proteins is modulated by phosphorylation. Reversible protein phosphorylation regulates multiple cellular processes, including metabolism, signal transduction pathways, cell cycle progression, oncogenic transformation, cell differentiation and apoptosis (Garcia et al., 2000; Garcia et al., 2003). Protein phosphorylation regulates the activities of protein kinases and protein phosphatases themselves (Hunter, 2000). Type 1 (PP1) and type 2A (PP2A) Ser/Thr protein phosphatases are major regulators of cell dephosphorylation. Binding of PP1 catalytic subunit (PP1c) to specific regulatory subunits generates a large family of PP1 holoenzymes (Cohen, 2002). For PP2A protein family, the catalytic PP2Ac subunit associates with a structural PP2A-A subunit to form a dimeric core enzyme that interacts with variable regulatory (B) subunit(s) (McCright et al., 1996) and confers substrate specificity to the dephosphorylating activity. The multiple PP1/PP2A holoenzymes are differentially expressed and targeted to distinct subcellular compartments.

Recent studies have identified small protein domains, termed protein transduction domains (PTDs) that promote the delivery of peptides and proteins into cells. The PTDs identified in proteins such as HIV-1 Tat, *Drosophila Antennapedia* homeoprotein (Antp) or HSV-1 VP22 can cross over the cell membrane by a still poorly defined process called protein transduction, and can reach the nucleus where they display their biological activity (Prochiantz, 2000). All known short cell-penetrating peptides derived from PTDs have a stretch of lysine or arginine-rich sequence required for intracellular delivery and peptides solubility. Since cellular delivery of conjugated (or fused) bio-molecules represents an interesting potential for drug design and molecular therapy (Ford et al., 2001), we have capitalized on these features to design peptides for intracellular delivery of phosphatase-targeting peptides.

We describe here a new phosphatase-derived drug technology (DPT), based on the concept of blocking the interaction of the PP1 or PP2A core catalytic subunit with their local regulatory partners using intracellular delivery of competing peptides in order to prevent specific substrate dephosphorylation. Several model systems have been investigated here. Based on structural analysis of PP2A interactions with casein kinase 2 alpha (CK2α) from various species and the SV40 virus encoded small t antigen, a common beta-strand structure interacting with the structural PP2A-A subunit was identified. A new

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cell penetrating sequence was identified in a *Theileria parva* CK2α and was used as a new non toxic shuttle for *ex vivo* and *in vivo* intracellular delivery of sequences interfering with intra-molecular PP2A or PP1 holoenzymes. We show here successful intracellular peptide delivery in several *ex vivo* systems, including *P. falciparum* malaria parasites, and *in vivo* chick embryos. In addition, and consistent with recent data concerning the role of PP1/PP2A proteins in apoptosis, we also found that intracellular delivery of sequences interacting with PP1c or PP2A Bα subunits by DPT-sh1 derived penetrating peptides induced cell death in several cell lines.

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MATERIAL AND METHODS

Cell culture and reagents

Adherent HeLa cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) as exponentially growing subconfluent monolayer on microplates or in 12, 24 or 96 wells plates. Jurkat lymphoid T cells (clone E6, ATCC) were cultured in RPMI 1640 glutamax medium (Gibco). Both cell lines were cultured in medium supplemented with 10% (v/v) fetal calf serum and antibiotics (100 U/ml streptomycin-penicillin). *P. falciparum* parasites (FUP/CB strain) were cultivated at 5% hematocrit in human A+ erythrocytes as described by Trager and Jensen (1976), in RPMI 1640 medium supplemented with 35 mM HEPES, 24 mM $NaHCO_3$, 5% human AB+ serum, 0,25% albumax, 1mg/mL hypoxanthine and 5mg/ mL gentamycin at 37°C.

Streptavidin-conjugated peroxydase (HRP), 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 3-4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. The annexin-V-fluorescent binding assay kit was from Roche.

Peptides

HPLC purified NH_2-biotinylated or FITC-conjugated peptides (purchased from Neosystem) were synthesized by solid-phase peptide synthesis. Peptides were dissolved in 150 mM NaCl and stored frozen at -20 °C until use.

PP2A proteins and antibodies

Protocol for purification of PP2A proteins (trimeric PP2A1 and PP2A-A or Bα subunits) from Pig brain was adapted from Waelkens et al. (1987). Characterization of PP2A antibodies (polyclonal guinea pig anti-A, or anti-B and anti-C subunits) has been previously described (Bosh et et al., 1995).

PP2A-binding assays on cellulose-bound CK2α and CD28 peptides

Overlapping dodecapeptides scanning the whole *T. parva* CK2α sequence or the CD28 intracytoplasmic domain were prepared by automated spot synthesis (Abimed, Langerfeld, Germany) onto an amino-derived cellulose membrane, as described (Frank and Overwin, 1996; Valle et al., 1999). Membrane was blocked using SuperBlock (Pierce), incubated with purified PP2A-A subunit or PP2A1 holoenzyme and after several washing steps,

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incubated with anti-PP2A antibody, followed by PO-conjugated secondary antibody. Positive spots were visualized using the ECL system.

Cellular localization of tagged peptides

Exponentially proliferating cells were rinsed twice in phosphate buffer saline (PBS). A total of 3 or 6.10^4 cells per well were seeded in 24 well plates and incubated in DMEM medium at 37°C. Twenty-four hours later, biotinylated peptides preincubated with streptavidin (ratio 1:4; 30 minutes at room temperature) were added to the cells and incubated for different periods of time at 37°C. Cells were rinsed twice in PBS, incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 5 min, washed in PBS and analyzed by microscopy. For FITC-conjugated peptides, cells were incubated with the peptides and washed twice in PBS as above, and then directly analyzed by fluorescence microscopy.

DPT-4 and DPT-5 biotinylated peptides were also directly incubated with cells for intracellular localization. Cells were then rinsed twice in PBS, fixed with 100% ethanol for 10 min. before adding 10 µg/ ml streptavidin-peroxydase. After a 30 min. incubation at 37°C, cells were rinsed twice in PBS, incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 5 min, washed in PBS and analyzed by microscopy.

For FITC-conjugated peptides, cells were incubated with the peptides and washed twice in PBS as above, and then directly analyzed by fluorescence microscopy.

Quantification of peptide internalisation

Prior to incubation, peptides were pre-incubated with Streptavidin-HRP conjugated in a 4:1 ratio. Cells at 50% of confluence were incubated with 10 µM or 100 µM of peptide in 24 wells plates. After 24 h, cells were washed twice in PBS and lysed in 300 µl of 0,1 M Tris pH 8; 0.5% NP-40 buffer, 10 minutes, on ice. Cellular debris were remove by centrifugation at 13000 g; 10 minutes; 4°C. A total of 50 µl of cell lysate were mixed with 50 µl of OPD buffer (51.4 mM Na_2HPO_4; 24.3 mM citric acid) then mixed with 100 µl of OPD solution (one OPD tablet from SIGMA in 100 ml OPD buffer in wich 40 µl of 30% hydrogen peroxide is added just prior use). After 10 to 20 minutes, reaction was stopped by addition of 100 µl HCl 1M and OD was read at 490 nm.

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Determination of peptide interaction with PP1 or PP2A targets

1.10^6 cells were washed twice then lysed 10 minutes on ice in 300 µl of lysis buffer (50 mM Tris pH7.4; 200 mM NaCl; 20% glycerol; 1% NP-40; 10 mM EDTA; 1 mM PMSF; 10 mM NaF; 1 mM orthovanadat; "complete, EDTA-free" protease inhibitor cocktail from Roche). Lysates were clarified at 13000 g; 10 minutes; 4°c; and were pre-incubated with biotinylated peptides at 100 µM; 1h; room temperature. Following this pre-incubation, 30 µl of streptavidin-coated immunomagnetic beads (Calbiochem) were added for 2h; 4°C. Biotinylated peptides were pulled down with streptavidin beads and washed four times in 750 µl of lysis buffer. Bound proteins and clarified lysates were then analysed by Western Blotting using PP1 or PP2A antibodies.

Cytotoxicity and Annexin-V assays

The cytotoxicity of peptides in HeLa or Jurkat cells lines was analyzed by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide named MTT for adherent cells (Mosmann, 1983), or MTS for lymphocytic cells (Hansen et al., 1989) as described by the manufacturer (Sigma).

For detection of apoptotic cells, we used an Annexin-V-APC conjugated kit (Roche) for the assessment of outer leaflet exposure of phosphatidylserine (PS) in the plasma membrane of apoptotic cells. Staining was performed according to the manufacturer's instructions. A total of 20.000 cells were analyzed by flow cytometry in a FACSCalibur cytofluometer (BD Biosciences). Necrotic cells were excluded by Propidium iodide (PI) staining, and single annexin V-positive cells were considered apoptotic.

Use of penetrating peptides in the chick embryo

Isa Brown chick eggs were incubated at 38°C and windowed on day 2 of incubation. Embryos were injected in the neural tube between the 10^{th} and 15^{th} somite under a dissecting microscope with 0,1-0,5µl 2 mM NH_2-biotinylated peptides in Hank's solution. After twenty-four hours, embryos were collected and directly frozen at –70°. Detection of NH2-biotinylated peptides on 30 µm cryosections was analyzed as described above for cellular assays.

Analysis of intracellular peptide delivery in *P. falciparum* infected human erythrocytes

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Fluorescein-labeled DPT-sh1 or DPT-sh2 were added at a 50 μM final concentration to uninfected erythrocytes control or a *P. falciparum* culture of plasmagel purified parasites Lambros and Vanderberg (1979) at 1% haematocrit in 24 well plates for 6 hours under 5% CO_2 atmosphere. Erythrocytes were washed twice in PBS. Parasite nucleus was stained using 5μg/ ml Hoechst 33342 (Molecular probes). DPT-sh1 and DPT-sh2 penetration was analyzed by fluorescence microscopy (Leica).

Bio-informatics and structure analysis

A homology model of bovine CK2α was constructed on the basis of the X-ray crystal structure of 1lr4 from the Brookhaven Protein Data Bank (PDB) (Berman et al., (2000). The sequences of the target and template protein were aligned using clustalX Thompson et al. (1997), and Modeller 4 (Fiser and Sali, (2003), was used to build a CK2α model. The model was refined by 100 steps of descent, followed by 200-300 steps of conjugate gradient energy minimization using InsightII (http://www.accelrys.com/insight/). The molecular assemblies between CK2α model (pdb 1b3u) and the X-ray crystal structure of PP2A-A subunit were then constructed by docking. The automated docking software Hex (http://www.biochem.abdn.ac.uk/hex/) with the macro molecular graphics package that uses a spherical polar Fourier correlation was used to accelerate docking calculations. After docking, energy minimization was applied on the whole complex with InsightII software. The figures were produced with GRASP (Nicholls et al., 1991).

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RESULTS

The DPT concept, illustrated in Figure 1, is based on intracellular delivery of specific peptides, interacting with Ser/Thr phosphatases PP1 or PP2A. We were first interested in the characterization of PP2A interacting sequences in CK2α proteins and we identified a new cell penetrating peptide. We took advantage of this result to generate hybrid CPPs containing a penetrating sequence derived from CK2α proteins fused to sequences interacting with PP1 or PP2A to assess their biological properties.

Structural analysis of PP2A interacting peptides in CK2α proteins

To identify CK2α peptides able to bind *in vitro* to PP2A, a series of 205 overlapping dodecapeptides from the *T. parva* CK2α protein was produced onto a cellulose membrane and incubated with purified PP2A holoenzyme or with PP2A subunits. Three overlapping peptides corresponding to a major docking site (named site 1) that binds to trimeric PP2A1 were detected (Figure 2A upper panel). This region is remarkably conserved among distinct CK2α proteins such as mammalian (human, bovine), or *falciparum* a parasite (Figure 2B), and represents a promiscuous CK2α PP2A interaction sequence. Interestingly, the KHENRKLYRKD sequence from the SV40 virus small t antigen that contains a PP2A docking site (Mateer et al., 1998), presents significant similarity with the sequence immediately preceding the CK2α site1 (Figure 2B). The peptide corresponding to residues 105-131 of SV40 small t antigen adopts a β-strand structure that is essential for high affinity interaction with the PP2A dimer and inhibits phosphatase activity (Mateer et al., 1998). Bio-informatics and structural analysis of CK2α indicated that site 1 is also an anti-parallel β-strand (Figure 2C and 2D). Surprisingly, when the CK2α peptide array was incubated with a purified PP2A-A subunit, two additional sites, (site 2 and site 3), were detected (Figure 2A lower panel). These new potential docking sites are located in the region of the ATP binding site.

Identification of new PP2A-interacting cell permeable peptides

All known short cell permeable peptides (CPPs) that translocate into eukaryotic cells are polycationic peptides containing 6-8 R and/or K residues. Since the CK2α sites 1-3 have a high R+K content, we hypothesized that they may represent a new source of CPP. To test this hypothesis, we chose to analyze the cell penetration potential of dodecamer

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#81 (VKKKKIKREIKI), which is part of site 3, that was called DPT-sh1 (table 1A).
At this time, we focused our interest on DPT-sh1 for its putative dual ability to interact with PP2A and to penetrate into cells due to its poly-basic sequence, as many known CPPs (table 1B). Biotinylated or FITC-labeled DPT-sh1 was used to investigate its ability to penetrate and to deliver a protein marker (human peroxydase) into lymphoid Jurkat or adherent HeLa cells. The amount of DPT-sh1 peptide internalized in Jurkat cells at 37°C was time- and concentration-dependent (Figure 3A). No uptake was detected at 4°C. In HeLa cells, FITC-tagged DPT-sh1 localized exclusively in the cytoplasm (Figure 3A lower panel) in contrast to most known CPPs that display a nuclear, or both nuclear and cytoplasmic localization. In this way, DPT-sh1 is of great interest to study regulation of cytoplasmic protein by PP2A or PP1. Furthermore, as a control of polycationic CPP with classical nuclear localization, we designed a new polycationic peptide named DPT-sh2 with an arbitrary polycationic penetrating sequence based on a trimeric repeat of RQKLRI. This sequence is closer to CK2α from *P. falciparum* than those from *T.parva* parasite (table 1A). Interestingly, DPT-sh2 (Figure 3B) delivered a protein marker (human peroxydase) linked through a biotin-streptavidin bridge into the cytoplasm of HeLa cells as efficiently as DPT-sh1. Moreover, DPT-sh2 displayed cytoplasmic and nuclear/nucleolar localization similar to Tat, a penetrating peptide derived from the viral transcription factor tat (Table 1B). Cell viability 48 hours after peptide penetration in HeLa or Jurkat cells was analyzed by the MTT or MTS colorimetric assay. No toxicity was observed in presence of DPT-sh1 even at concentrations up to 500 µM (Figure 3C). In addition, as shown in figure 3D, internalisation of FITC-conjugated DPT-sh1 and DPT-sh2 peptides in human red blood cells was undetectable. Surprisingly, red blood cells infected with the human malaria parasite *Plasmodium falciparum* internalized the DPT-sh1 peptide. Further analysis indicated that fluorescence was restricted to parasite, as confirmed by Hoechst staining of *P. falciparum* nucleus (data not shown). The FITC-labelled DPT-sh1 mostly localized to the cytoplasm of pigmented parasites, but also in merozoites prior to erythrocyte disruption. Similar uptake and localization were found with DPT-sh2 (data not shown).

Furthermore, as shown in figure 3E, based on internalisation of streptavidin-HRP conjugated molecules by biotinylated peptides, we quantified in HeLa cells the cargo effect of DPT-sh1 and DPT-derived peptides in comparison to a HIV Tat-derived penetrating peptide. We observed that in contrast to DPT-sh1 alone, DPT-derived

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peptides displayed about 50% at 10 μM and equivalent efficiency of penetration as Tat alone at 100 μM.

In vivo analysis: Use of DPT-sh1 shuttle in chick embryos

In order to test the *in vivo* effect of the penetrating peptides, we injected biotinylated DPT-sh1 into the chick embryo. The peptide was injected at a 2 mM concentration into the neural tube of two days chick embryos, and 54 control embryos were injected with Hank's solution. Even at such a high concentration, the peptide showed limited toxicity, as the survival rate was similar (about 63%) in both groups. Peptide penetration was rapid, and after 24 hours, the localisation was restricted caudally to the 30^{th} somite. Cryosections analysed with streptavidin-peroxydase showed that peptides were present predominantly in the notochord but also in the neural tube, and in a dorsolateral region of the somitic mesoderm underlying the ectoderm (Figure 4).

Taken together, these data indicate that DPT-sh1 and DPT-sh2 are new non toxic cell penetrating peptides.

Effect of intracellular delivery of a peptide that binds in vitro to the PP2A-Bα subunit

The intra-cytoplasmic tail of the T lymphocyte surface receptor CD28 contains 41 residues that can associate with several members of the PP2A family (Chuang et al., 2000). To identify CD28 sequences able to bind the PP2A-Bα subunit, overlapping dodecapeptides scanning the cytoplasmic tail were produced onto a cellulose membrane and incubated with purified PP2A proteins. Two PP2A-Bα interacting peptide spots with an overlapping sequence (residues 196-210) were identified (Figure 5A). The corresponding soluble peptide, called DPT-4 was produced, as well as DPT-5, which combines DPT-sh1 and DPT-4 sequences (see Table 1A). Using FITC-tagged peptides, we showed that DPT-5, but not DPT-4 was able to penetrate within HeLa cells and localize in the cytoplasm and the nucleus (Figure 5 A). Furthermore, as expected, pull-down experiments confirmed that DPT-4 and DPT-5 interacted with PP2A-Bα subunit (Figure 5B). In addition, DPT-5 but not DPT-4, induced loss of cell viability (Figure 5C). In Jurkat cells, DPT-5 penetrated into the cell and provoked apoptosis, whereas DPT-4 did not penetrate and was unable to induce apoptosis (Figure 5D). These data suggest that intracellular delivery of a PP2A-Bα interacting motif is sufficient to affect cell viability.

Effect of intracellular delivery of a PP1c binding consensus motif

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We recently identified a new PP1c docking consensus (F-xx-R/K-x-R/K) motif in anti-apoptotic Bcl-x_L (Ayllon et al.,2002) and in pro-apoptotic Bad (Garcia et al., 2004), two members of the Bcl-2 family. Since apoptosis induced by interleukin-2-deprivation in murine TS1αβ cells requires activation of a PP1 Bad-associated phosphatase activity (Ayllon et al., 2000), we reasoned that intra-cytoplasmic delivery of a peptide containing the PP1c docking motif from Bad should interfere with cell death induction. We designed DPT-6, a peptide sequence corresponding to the dual repeat of PP1c docking motif from Bad (see Table 1A). Despite containing 5 Arg residues, DPT-6 was unable to serve as a CPP and penetrate into HeLa cells (Figure 6A, left). However, DPT-7 which combines DPT-sh1 and the DPT-6 sequences (see Table 1B), penetrated and localized in the cytoplasm of HeLa cells (Figure 6A, right). Consistent with this, DPT-7, but not DPT-6 provoked cell death in Hela and Jurkat cells (Figure 6B and C). The invariant position of the amino-acid F in the PP1c consensus docking motif was shown to be critical (Ayllon et al., 2000; Garcia et al., 2004). Interestingly, DPT-9, which carries a F to A mutation in both PP1c consensus docking motifs, no longer exhibited any toxic effect (while still penetrating into the cell). Peptide DPT-8 with a single mutation abrogating the first PP1c consensus docking motif still inhibited cell viability (Figure 6B). Furthermore pull-down experiments confirmed that DPT-6 and DPT-7 but not DPT-9 interacted with PP1c subunit (Figure 6D). These data suggest that the presence of at least one PP1c docking motif is sufficient to affect cell viability.

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DISCUSSION

We described here new experimental evidences providing proof of principle for the Drug Phosphatase Technology. This novel concept for rational drug design and potential therapy aims at targeting the families of PP1/PP2A holoenzymes, which control a major portion of serine/threonine phosphatase activity in cell extracts. As illustrated in Figure 1, phosphatase-derived drug technology, DPT, is based on cell delivery of PP1/PP2A interacting peptides.

DPT-derived peptides : tools to deregulate intracellular pathways

Our results suggest that PP2A is a source for new biologically active or inactive cell penetrating peptides. Small cationic cell penetrating peptides derived from the human immunodeficient virus (HIV)-1 Tat and *Drosophila Antennapedia* homeoprotein (Antp) have been used to introduce multiple molecules, including synthetic small molecules, peptides and proteins into cells and also animal models (Schwarze et al.,1999). In addition, non naturally CPP corresponding to chimeras containing charged residues such as MGP (Morris et al., 1999), a chimera between SV40 NLS and GP41, have also been characterized (table 1B). We have clearly identified polycationic peptides located within PP2A-binding sequences of CK2α proteins. Bio-informatics and structural analysis suggested that both SV40 small t and CK2α proteins, two previously characterized PP2A interacting proteins, share a putative common β-strand structure interacting with the structural PP2A-A subunit. This β-strand structure favours heteromeric interaction within the trimeric PP2A1 holoenzyme. In contrast, the sequence corresponding to DPT-sh-1 permeable peptide is localized in a poorly accessible α helix within CK2α (Figure 2C and 2D). Within the cell, the PP2A-A protein is recruited into two distinct dimeric and trimeric pools of PP2A holoenzyme and never has been found as a free subunit (Ruediger et al., 1997). These observations suggest that interaction between PP2A-A subunit and DPT-sh1 revealed by *in vitro* spot assay is not favoured *in situ* and may explain the absence of DPT-sh1 toxicity illustrated in Figure 3. The cytoplasmic localisation of DPT-sh1 itself or associated with a protein is an unusual feature in CPP designed from naturally occurring sequences. Indeed Tat, Antp and various arginine rich peptides mainly localize within the nucleus (Table 1B). We proposed to use DPT-sh1 as a new non toxic peptide shuttle to interfere with PP1/PP2A-dependent apoptotic pathways. The lack of toxicity and the rapid delivery of

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protein-bound–DPT-sh1and DPT-sh2 respectively into the cytoplasm and into the nucleus represent two attractive features for their potential use in basic research or/and in therapeutic approach. In this context, our experiments in chick embryos indicate that DPT-sh1 micro-injection in neural tube will be useful to interfere with regulatory pathways in vertebrate development.

PP2A, previously named polycation stimulated phosphatase, is known to be activated by polycationic proteins such as protamines (Waelkens et al., 1987), suggesting that polycationic peptides derived from PP2A interacting proteins such as CK2α and protamin, may be used as new CPPs in DPT-technology. Consistent with this notion, previous reports indicate that Vpr, a 96 amino acid protein encoded by HIV-1, can penetrate and induce apoptosis in different types of human cell lines. Interestingly, this effect requires the presence of a short C-terminal domain containing the conserved sequence HFRIGCRHSRIG. Several CPPs containing this sequence penetrate and provoke apoptosis in human cells (Arunagiri et al., 1997). Interestingly we have recently identified by *in vitro* experiments a PP2A binding region within this domain (Godet et al. manuscript in preparation).

Our recent results suggested that PP1 is a key phosphatase player in apoptosis (for review see Garcia et al., 2003). The simultaneous presence in most PP1 interacting proteins of two distinct PP1c consensus docking motifs, R/K-x$_{(0,1)}$-V-x-F and F-xx-R/K-x-R/K, also identified in some anti-apoptotic members of the Bcl-2 family, led us to propose a new concept of PP1 predictive signature (for details see Garcia et al., 2004) and also web site : http://pp1signature.pasteur.fr). We hypothesized that the bioinformatics identification of PP1c docking sites in putative medically important PP1c interacting proteins combined to the use of DPT-shuttles and/or non toxic CPPs, may be relevant to develop DPT. We have previously shown that growth factor deprivation-induced apoptosis operates by regulating Bad-phosphatase-PP1 activity in murine interleukin-4 dependent T lymphocyte (Ayllon et al., 2002) and we also characterized *in vitro* a PP1c docking motif in Bad (Garcia et al., 2004). This system was used to carry out proof of principle experiments for this strategy. Indeed as reported in Figure 6, introduction of PP1c-interacting motif, deduced from Bad interacting sequence, triggered cell death in HeLa and Jurkat cells.

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The DPT concept is a new approach for drug discovery against tumours and infections

Concerning cancer, within the three last decades, the major technical progress in molecular biology combined with the discovery of oncogenes, and more recently the identification of apoptotic factors, open a way to rational drug design. The better understanding of signalling pathways driven by Ras oncogen and by protein kinases or phosphatases allowed the development and use of small inhibitory molecules. Consequently, distinct farnesyl transferase inhibitors, initially designed as a potential anti-ras therapy or different specific pharmacological inhibitors of tyrosine kinases or antisense therapy against PKC or PKA have been already used in clinical trials (Liu et al., 1998; Zhang, et al., 1999; Yuen et al., 1999). However, appropriate utilization of these new molecules alone or in combination with chemotherapy is still mater of debate.

In this context how PP1/PP2A protein phosphatases and DPT may help to design more specific and less toxic new cancer drugs ? It is established that viruses have developed specific pro and anti-transforming strategies to deregulate cells *via* PP2A. Interaction of PP2A-A subunit with small t antigen encoded by transforming *papovae* viruses activates MEK/ERK, two major components of the ras transforming pathway (Sontag et al., 1997). In contrast, interaction between PP2A-Bα subunit and adenoviral protein E4orf4 induces cancer-cell specific apoptotic pathways which, depending on the cell type, operates trough caspase-independent or caspase-dependent apoptosis (Shtrichman et al., 1999). Given that interaction of E4orf4 with PP2A-Bα regulatory subunit induces apoptosis of transformed cells in a p53-independent manner (Livne et al., 2001), we speculated that peptides interacting with PP2A-Bα may also induce apoptosis. Consistent with this hypothesis, intracellular delivery of PP2A-Bα interacting sequences deduced from CD28 antigen in the PP2A-Bα-interacting DPT-5 peptide, clearly provoked cell death. This result suggest that DPT-derived peptides mimicking signalling generated by E4orf4-PP2A interaction could represent a new creative approach to counteract resistance of cancer cells against drug-induced apoptosis. Contrary to PP1 interacting subunit, the motifs required for PP2A interaction are not well characterized since no consensus is clearly established. Bio-informatics studies are under progression to elucidate this mystery. Data obtained from bio-informatics will give us some clues to design PP2A interacting peptides mutants in order to validate the specificity of such peptides as DPT5. This work illustrates the relevance of targeting regulatory sites and not only catalytic motifs of PP1 and PP2A, in order to specifically induce apoptosis of tumour or infected

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cells. We now are pursuing this work by crystallography studies in order to understand how DPT peptides interact with PP1 and PP2A compared to the full length proteins. In this way, the use of CPPs, especially when targeting PP1 and PP2A, is much more relevant than the use of known pharmacological small molecules that directly inhibit the catalytic function in a toxic way for all non transformed and transformed cells.

Recently, two breakthrough studies upgrade the interest of both small molecules and penetrating peptides for rationale drug design. First a recent work by Plescia et al. (2005) describes a penetrating peptide based on tat penetrating sequence fused to 9 aa deduced from survivin sequence. This peptide, named Shepherdin, is the first non-small molecule inhibitor of Hsp90 and is shown to inhibit tumour growth in mice and to be very well tolerated *in vivo* suggesting that peptidomimetic inhibitors of Hsp90 may circumvent some of the toxicity issues currently limiting the clinical utility of small molecule Hsp90 inhibitors. The second concerns small molecules, based on chemical biology approach Boyce et al. (2005) identified salubrinal as a small molecule that protects against ER-stress-induced cell death~~,~~ through its ability to prevent dephosphorylation of eIF2 alpha by PP1. Salubrinal also blocks eIF2alpha dephosphorylation mediated by a herpes simplex virus protein and inhibits viral replication. This paper shows that it is possible to target dephosphorylation of selected protein phosphatase substrates for future therapeutic development."

Concerning infection, we focused on malaria because the absence of vaccine and the emergence of drug-resistant malaria parasites represents an important public health problem in sensitive countries. Since there is no evidence for endocytosis in non infected mature erythrocyte (Kirk, 2001), the selective penetration of DPT-shuttles in red cells infected by *P. falciparum* opens the way to a new drug therapy. In this context, pharmacological inhibition of PKA, as well as abrogation of PP1 expression by short interfering RNA (siRNA), led to inhibition of the parasite intraerythrocytic development (Syin et al., 2001; Kumar et al., 2002). Interestingly, our data included in the paper submitted by Guergnon *et al*. demonstrated that DPT-PKI, a new specific PKA inhibitor that combines DPT-sh1 and the PKI sequences defined by Scott et al., (1985), inhibits *Theileria annulata*-directed PKA survival pathway in parasitized bovine lymphocytes, suggesting that it is useful for cell culture experiments and to develop novel therapies based on PKA modulation. This tool is under evaluation in both cancer and *P. falciparum* parasites.

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References

Arunagiri C, Macreadie I, Hewish D, and Azad A (1997) A C-terminal domain of HIV-1 accessory protein Vpr is involved in penetration, mitochondrial dysfunction and apoptosis of human CD4+ lymphocytes. *Apoptosis* **2:** 69-76.

Ayllon V, Martinez AC, Garcia A, Cayla X, and Rebollo A (2000) Protein phosphatase 1alpha is a Ras-activated Bad phosphatase that regulates interleukin-2 deprivation-induced apoptosis. *EMBO. J* **19:** 2237-2246.

Ayllon V, Cayla X, Garcia A, Fleischer A, and Rebollo A (2002) The anti-apoptotic molecules Bcl-xL and Bcl-w target protein phosphatase 1α to Bad. *Eur J Immunol* **32:** 1847-1855.

Berman HM, Bhat T.N, Bourne P.E, Feng Z, Gilliland G, Weissig H, and Westbrook J (2000) The Protein Data Bank and the challenge of structural genomics *Nat Struct Biol* **7** ***Suppl :*** 957-959.

Bosch M, Cayla X, Van Hoof C, Hemmings B, Ozon R, Merlevede W, and Goris J (1995) The PR55 and PR65 subunits of protein phosphatase 2A from *Xenopus laevis*: molecular cloning and developmental regulation of expression *Eur. J. Biochem.* **230:** 1037-1045.

Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, Kaufman RJ, Ma D, Coen DM, Ron D, Yuan J. (2005) A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. *Science* **307**:935-939.

Chuang E, Fisher TS, Morgan RW, Robbins MD, Duerr JM, Vander Heiden MG, Gardner JP, Hambor JE, Neveu MJ, and Thompson CB (2000) The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. *Immunity* **13:** 313-322.

Cohen PT.(2002) Protein phosphatase 1-targeted in many directions. *J Cell Sci* **115:** 241 - 256.

McCright B, Rivers AM, Audlin S, and Virshup DM (1996) The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. *J Biol Chem* **271:** 22081-22089.

Fiser A, and Sali A (2003) Modeller: generation and refinement of homology-based protein structure models. *Methods Enzymol* **374:** 461-491.

Ford KG, Souberbielle BE, Darling D, and Farzaneh F. (2001) *Gene Ther* **8:** 1-4.

Frank R, and Overwin (1996) H SPOT synthesis *Methods Mol Biol* **66:** 149-169.

Garcia A, Cayla X, and Sontag E (2000) Protein phosphatase 2A: a definite player in viral and parasitic regulation. *Microbes Infect* **2:** 401-407.

Garcia A, Cayla X, Guergnon J, Dessauge F, Hospital V, and Rebollo MP, Fleischer A, Rebollo A (2003) Serine/threonine protein phosphatases PP1 and PP2A are key players in apoptosis. *Biochimie* **85:**721-726.

Garcia, A., Cayla, X., Caudron, B., Deveaud,E., Roncal, F., and Rebollo, A. (2004) New insights in protein phosphorylation: a signature for Protein Phosphatase 1 interacting proteins. C.R. Biologies *C.R. Biologies* **327:** 93-97.

Hansen, M.B., Nielsen, S.E., Berg, K. (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* **119:** 203-210.

Hunter, T. (2000) Signaling-2000 and beyond. *Cell* **100:** 113-127.

Kirk, K. (2001) Membrane transport in the malaria-infected erythrocyte. *Physiol Rev* **81:** 495-537.

Kumar R, Adams B, Oldenburg A, Musiyenko A, and Barik S (2002) Characterisation and expression of a PP1 serine/threonine protein phosphatase (PfPP1) from the malaria parasite, *Plasmodium falciparum*: demonstration of its essential role using RNA interference. *Malar. J* **2:** 1-11.

Lambros C, and Vanderberg JP (1979) Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* **65:** 418-420.

Livne A, Shtrichman R, Kleinberger T (2001) Adenovirus type 5 E4 open reading frame 4 protein induces apoptosis in transformed cells. *J Virol* **75:** 789-798.

Liu M, Bryant MS, Chen J, Lee S, Yaremko B, Lipari P, Malkowski M, Ferrari E, Nielsen L, Prioli N, Dell J, Sinha D, Syed J, Korfmacher WA, Nomeir AA, Lin CC, Wang L, Taveras AG, Doll RJ, Njoroge FG, Mallams AK, Remiszewski S, Catino JJ, Girijavallabhan VM, Bishop WR (1998) Antitumor activity of SCH 66336, an orally bioavailable tricyclic inhibitor of farnesyl protein transferase, in human tumor xenograft models and wap-ras transgenic mice. *Cancer Res* **58:** 4947-4956.

Mateer SC, Fedorov SA, and Mumby MC (1998) Identification of structural elements involved in the interaction of simian virus 40 small tumor antigen with protein phosphatase 2A *J Biol Chem* **273:** 35339-35346.

Morris MC, Chaloin L, Méry J, Heitz F, and Divita G (1999) A novel potent strategy for gene delivery using a single peptide vector as a carrier. *Nucleic Acids Res* **27:** 3510–

3517.

Mosmann, (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65:** 55-63.

Nicholls A, Sharp KA, and Honig B (1991) Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* **11:** 281-296.

Plescia J, Salz W, Xia F, Pennati M, Zaffaroni N, Daidone MG, Meli M, Dohi T, Fortugno P, Nefedova Y, Gabrilovich DI, Colombo G, Altieri DC. (2005) Rational design of shepherdin, a novel anticancer agent. *Cancer Cell* **5** : 457-468.

Prochiantz A (2000) Messenger proteins: homeoproteins, TAT and others *Curr Opin Cell Biol* **12:** 400-406.

Ruediger R, Brewis N, Ohst K, and Walter G (1997) Increasing the ratio of PP2A core enzyme to holoenzyme inhibits Tat-stimulated HIV-1 transcription and virus production *Virology* **238:** 432-443.

Schwarze SR, Ho A, Vocero-Akbani A, and Dowdy S.F. (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* **285:**1569-1572.

Shtrichman R, Sharf R, Barr H, Dobner T, and Kleinberger T (1999) Induction of apoptosis by adenovirus E4orf4 protein is specific to transformed cells and requires an interaction with protein phosphatase 2A *Proc Natl Acad Sci U S A* **96:** 10080-10085.

Scott JD, and Fischer EH, Takio K, Demaille JG, and Krebs EG. (1985) Amino acid sequence of the heat-stable inhibitor of the cAMP-dependent protein kinase from rabbit skeletal muscle.*Proc Natl Acad Sci U S A* **82:** 5732-5736.

Sontag E, Sontag JM, Garcia A (1997) Protein Phosphatase 2A is a critical regulator of protein kinase C ζ signaling targeted by SV40 small t to promote cell growth and NF-κB activation. *EMBO. J.* **16:** 5662-5671.

Syin C, Parzy D, Traincard F, Boccaccio I, Joshi MB, Lin DT, Yang XM, Karine Assemat K, Doerig C, and Langsley G (2001) The H89 cAMP-dependent protein kinase inhibitor blocks *Plasmodium falciparum* development in infected erythrocytes. *Eur J Biochem* **268:** 4842-4849.

Thompson JD, Gibson, TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools *Nucleic Acids Res* **25:** 4876-4882.

Trager W, and Jensen J (1976) Human malaria parasite in continuous culture. *Science* **193:** 673-675.

Valle M, Munoz M, Kremer L, Valpuesta JM, Martinez AC, Carrascosa JL, Albar, J (1999) Selection of antibody probes to correlate protein sequence domains with their structural distribution. *Protein Sci* **8:** 883-889.

Waelkens E, Goris J, and Merlevede W (1987) Purification and properties of polycation-stimulated phosphorylase phosphatases from rabbit skeletal muscle *J Biol Chem* **262:** 1049-1059.

Zhang L, Lau YK, Xia W, Hortobagyi GN, and Hung MC (1999) Tyrosine kinase inhibitor emodin suppresses growth of HER-2/neu-overexpressing breast cancer cells in athymic nude mice and sensitizes these cells to the growth inhibitory effect of paclitaxel. *Clin. Cancer Res* **5:**, 343-353.

Yuen AR, Halsey J, Fisher G A, Holmlundg JT, Geary R S, Kwoh T.J, Dorr A, Sikic BI (1999) Phase I study of an antisense oligonucleotide to protein kinase C- alpha (ISIS 3521/CGP 64128A) in patients with cancer. *Clin. Cancer Res* **5:** 3357-3363.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1 : ***Schematic illustration of the DPT concept.***

DPT consists in intracellular delivery of bi-partite peptides including a short cell penetrating peptide fused to a peptide binding to the catalytic subunit of PP1 or to PP2A core or to holoenzymes. PP1/PP2A interacting proteins are represented as (i) and the phosphatase catalytic structures (PP1c or core PP2A) are schematically represented as (C). This strategy derives from two lines of results: i) identification of non toxic shuttles derived from CK2α. Depending on the sequence, this shuttle (grey rectangle) localizes in the cytoplasm (DPT-sh1) or similarly to other CPPs both in nucleus and cytoplasm (DPT-sh2); ii) identification of docking sites (black rectangles) in PP1- or PP2A-binding proteins. A predictive signature for PP1 binding motifs has been published (Garcia et al., 2004). Signatures for PP1 are available at http ://pp1signature.pasteur.fr.

Figure 2: ***PP2A binding assay on cellulose-bound CK2α peptides and structural analysis of PP2A interacting peptides***

(A) Autoradiogram of the PP2A binding assay on cellulose-bound CK2α peptides. 205 overlapping dodecapeptides with a two-amino acid shift scanning the entire *T. parva* CK2α sequence were synthesized on a cellulose membrane. The membrane was incubated with PP2A1 holoenzyme (upper panel) or PP2A-A subunit (lower panel) and subsequently with anti-PP2A antibodies followed by peroxydase-labelled anti-mouse antibodies. The interacting CK2α–derived peptides are boxed and the corresponding protein sequence is indicated. **(B)** Sequence homology of the PP2A binding sequence of SV40 virus small t antigen and site 1 PP2A binding sequence from *T parva* CK2α and homologous CK2 regions from various species. **(C)** Localisation of the PP2A binding site and DPT-sh1 peptide on a ribbon representation deduced from the CK2α crystal structure (left) and on the charge density on the surface of electrostatic potential (right). Both figures are shown in the same orientation. The PP2A binding site (red) is located in the β anti-parallel β-strand comprised between residues 165 and 184. The DPT-sh1 sequence (blue), which encompasses residues 257-272 is located in the aı α-helix. The electrostatic potential (red, -12 kTe-1; blue, +12 kTe-1) on the accessible surface of CK2α. The PP2A binding site and DPT-sh1 are on opposite sides of the molecule (circles) and electrostatic potentials. **(D)** Model (pdb 1b3u) of CK2α(coloured in blue) and PP2A-A (coloured in red) binding. The residues predicted to take part to binding are

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represented as surfaces. The DPT-sh1 peptide is coloured in yellow. The figure was created using the InsightII software.

Figure 3: ***Effect of peptides derived from PP2A interacting proteins on cell penetration, intracellular delivery of streptavidin-peroxydase and cell viability***

(A) Internalization of DPT-sh1 in HeLa and Jurkat cells Upper panel: Dose-dependence internalisation was measured after a 60 min incubation at 37°C. Middle panel: Kinetics of 10 µM DPT-sh1 cellular uptake by Jurkat cells at 37° C. Lower panel: Visualisation of FITC-tagged DPT-sh1 peptide uptake by HeLa cells using epifluorescence. HeLa cells were incubated with 10 µM peptide for 1 hour at 37° C and rinsed twice with PBS before fluorescence microscopy analysis. **(B)** Intracellular delivery of streptavidin-peroxydase by biotinylated-DPT-sh1, DPT-sh2 and TAT in HeLa cells. Streptavidin-peroxydase coupled with biotinylated peptides were incubated for 6 hrs at 37 °C and internalised complexes were visualised as indicated in Materials and Methods. **(C)** Cell viability assay. HeLa cells were incubated 48 hours with previous peptides prior to be processed for MTT staining.(**D**) Selective penetration of fluorescein-labeled DPT-sh1 peptide in *P. falciparum* FUP/CB infected erythrocyte: Non infected human erythrocytes (upper panels:) or *P. falciparum* infected human erythrocytes (lower panels) were analysed by light transmission (left panels) and fluorescent staining (right panels).**(E)** Quantification of internalised peptides in HeLa cells. DPT-sh1, DPT-5, DPT-7 and DPT-9 were incubated 24 h at 10 or 100 µM and compared to the well characterised penetrating peptide derived from HIV-1 TAT protein. This figure corresponds to a representative experiment repeated three times.

Figure 4: ***Localisation of biotinylated DPT-sh1 injected in the neural tube of 2 days chick embryos***

(A) Schematic representation of the section of three days old chick embryo presented in B and D. **(B)** Twenty four hours after injection in the neural tube (see Materials and Methods), the biotinylated DPT-sh1 peptide was detected on cryosections in the neural tube, in a dorsolateral region of the somitic mesoderm underlying the ectoderm, and in the notochord. **(C)** Enlargement of B shows the predominant signal in the notochord. **(D-E)** Absence of labelling on sections of control embryo injected with the Hank's solution alone. Bar: 30 mm.

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Figure 5: ***Effect of intracellular delivery of a peptide mimicking a PP2A docking sequence in CD28 antigen***

(A) membrane with overlapping dodecapeptides deduced from the short 41 residue intra-cytoplasmic CD28 sequence was incubated with purified Bα, A subunits or with PP2A1 holoenzyme and revealed as in figure 1A. Left panel shows the spots of interaction of CD28 with purified Bα subunit. Similar results were obtained with PP2A1 holoenzyme, which contains the Bα subunit (not shown). Right panel, HeLa cells were incubated with 10 µM FITC-tagged DPT-4 or DPT-5 peptides for 1 hr, rinsed and fixed with mowiol before fluorescence microscopy analysis. (**B**) Identification of lysates (10%) or bound proteins (50%) in pull-down experiments was realised by immunoblotting using antibodies against PP2A-Bα̃

(**C**) HeLa cells were incubated 48 hrs with peptide prior to be processed for detection MTT staining. DPT-sh1 peptide was used as control (data not shown). **(D)** Jurkat cells were treated with FITC-tagged DPT-4 or DPT-5 peptides for 24 hours in microplates. After treatment, cells were washed twice with PBS and stained with Annexin-V-APC. Cell death was measured by flow cytometry.

Figure 6 : ***Effect of intracellular delivery of a peptide mimicking the PP1 docking sequence of the pro-apoptotic protein Bad***

(A) HeLa cells were incubated with 10 µM FITC-tagged DPT-6 or DPT-7 peptides for 1 hour prior to be rinsed twice with PBS and analysed by fluorescence microscopy. **(B)** HeLa cells were incubated 48 hours with peptide prior to be processed for MTT staining detection. **(C)** Jurkat cells were treated with FITC-tagged DPT-6 or DPT-7 peptides for 24. Cells were then rinsed twice with PBS and stained with Annexin-V-APC. Cell death was assessed by flow cytometry. (**D**) Identification of lysates (10%) or bound proteins (50%) in pull-down experiments was realised by immunoblotting using antibodies against PP1c.

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Table 1: Conventional and new cell penetrating peptides containing PP1/PP2A-interacting motifs

Table 1 A : *Origin and sequence of DPT-peptides*

Protein origin	**Peptide sequence***	**acronym**
CKα (T.parva)	VKKKKIKREIKI	DPT-sh1
Synthetic sequence	RQKRLIRQKRLIRQKRLI	DPT-sh2
Protamin	RRRRRRRSRGRRRRTY	DPT-3
CD28 antigen	PRRPGPTRKHYQPYA**	DPT-4
CKα (T.parva) + CD28 antigen	VKKKKIKREIKI-PRRPGPTRKHYQPYA	DPT-5
Bad	**F**RG**R**S**R-F**RG**R**S**R** ***	DPT-6
CKα (T.parva) + Bad	VKKKKIKREIKI-**F**RG**R**S**R F**RG**R**S**R**	DPT-7
CKα (T.parva) + Bad mutant-1	VKKKKIKREIKI-**A**RG**R**S**R F**RG**R**S**R**	DPT-8
CKα (T.parva) + Bad mutant-2	VKKKKIKREIKI-**A**RG**R**S**R**A**R**G**R**S**R**	DPT-9

***** Single letter code for amino acids is used for all peptides**.**

****** This peptide binds *in vitro* to Bα but not to PP2A-A subunits

******* residues corresponding to unvariable amino-acids in PP1c site are in black and bold (mutation in red bold).

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Table 1 B *: Origin, sequence and biological properties of some known cell penetrating peptides*

Acronym	Sequence*	Cell Penetration		Cytoxicity	Protein carrier	
		cytoplasm	*nucleus*		*cytoplasm*	*nucleus*
TAT	YGRKKRRQRRR	+/-	+	+	+/-	+
ANTp	RQIKIWFQNRRMKWKK	+/-	+	+/-	+/-	+
****Transportan**	GWTNLSAGYLLGKINLKALAALAKK	+/-	+	nd	+/-	+
****MGP**	GALFLGFLGGAAGSTMGAWSQPKSK	+/-	+	nd	+/-	+
VPR	HFRIGCRHSRIG	+/-	+	+++	nd	nd
VPR	HFRIGCRHSRIGVTRQRRARNGASRS	+/-	+	+++	nd	nd***

*****Single letter code for amino acids is used for all peptides.

****Non naturally occuring CPPs

*****We have recently identified a new CPP binding PP2A-A and PP2A1 holoenzyme that contains part of this sequence and can carry 100 KDa proteins (Godet et al. in preparation).

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Table 1C : *Biological properties of DPT-peptides*

Peptide	***In vitro* binding** (spot assay)			**Cell binding** (pull down assay)			**Protein carrier**	**Apoptosis**
	PP1c	*PP2A-A*	*PP2A Bα*	*PP1c*	*PP2A-A*	*PP2A Bα*	*(HRP assay)*	
DPT-sh1	nd	+	-	-	-	-	+	-
DPT-sh2	nd	nd	nd	nd	nd	nd	+	-
DPT-3	nd	nd	nd	nd	nd	nd	+	+
DPT-4	-	-	+	nd	nd	+	-	-
DPT-5	nd	nd	nd	nd	nd	+	+	+
DPT-6	*****+	nd	nd	+	nd	nd	-	-
DPT-7	nd	nd	nd	+	nd	nd	+	+
DPT-8	nd	nd	nd	nd	nd	nd	+	+
DPT-9	***-**	nd	nd	-	nd	nd	+	-

*The spot assays involving PP1c consensus docking sequences, used in wt (DPT6) or mutated (DPT9) peptides has been previously published (Garcia et al., 2004).

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Figure 1

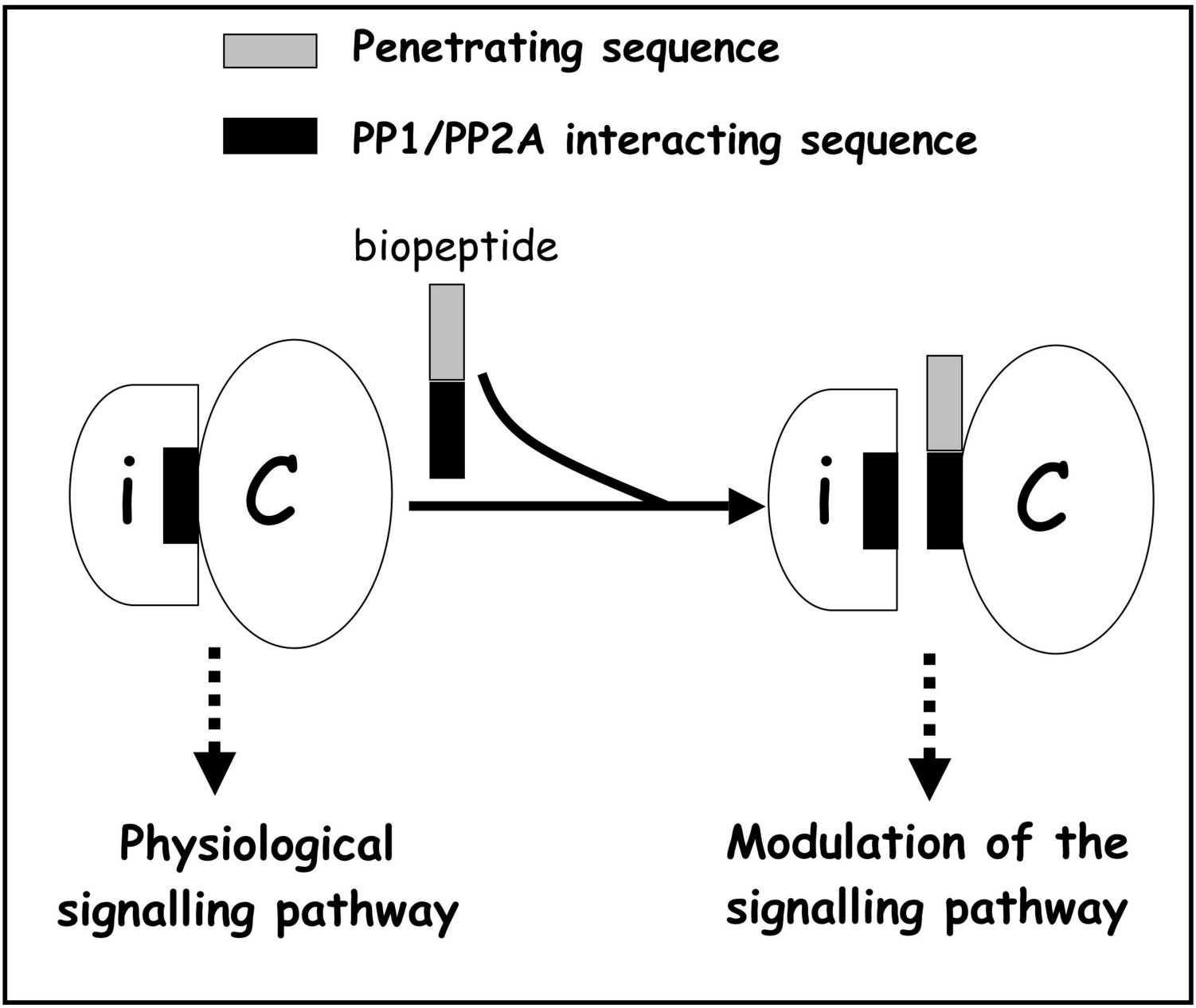
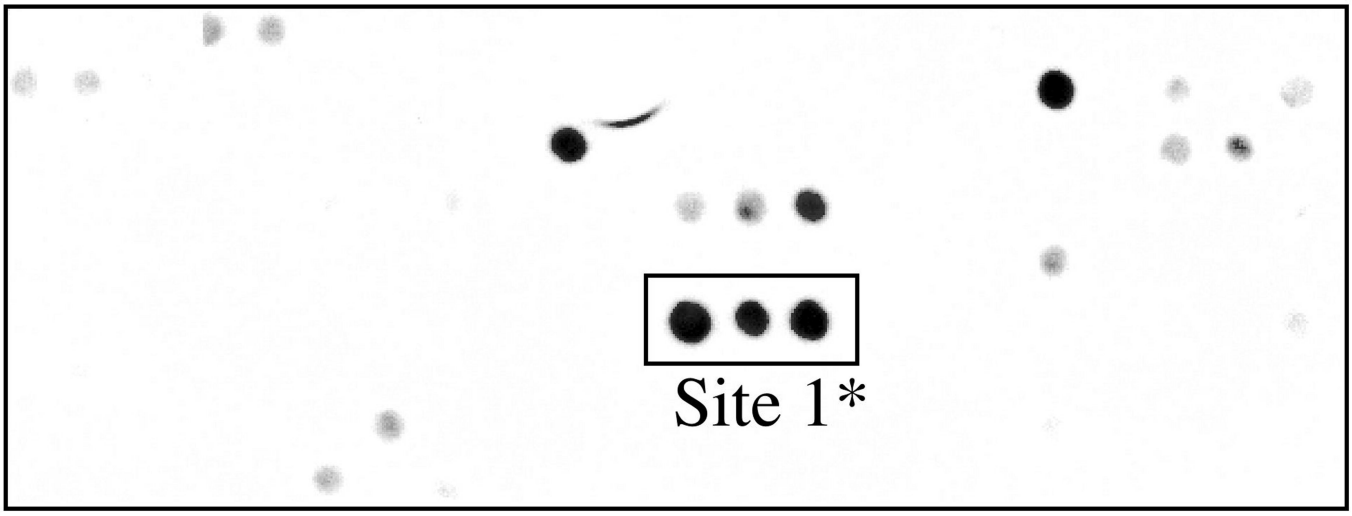
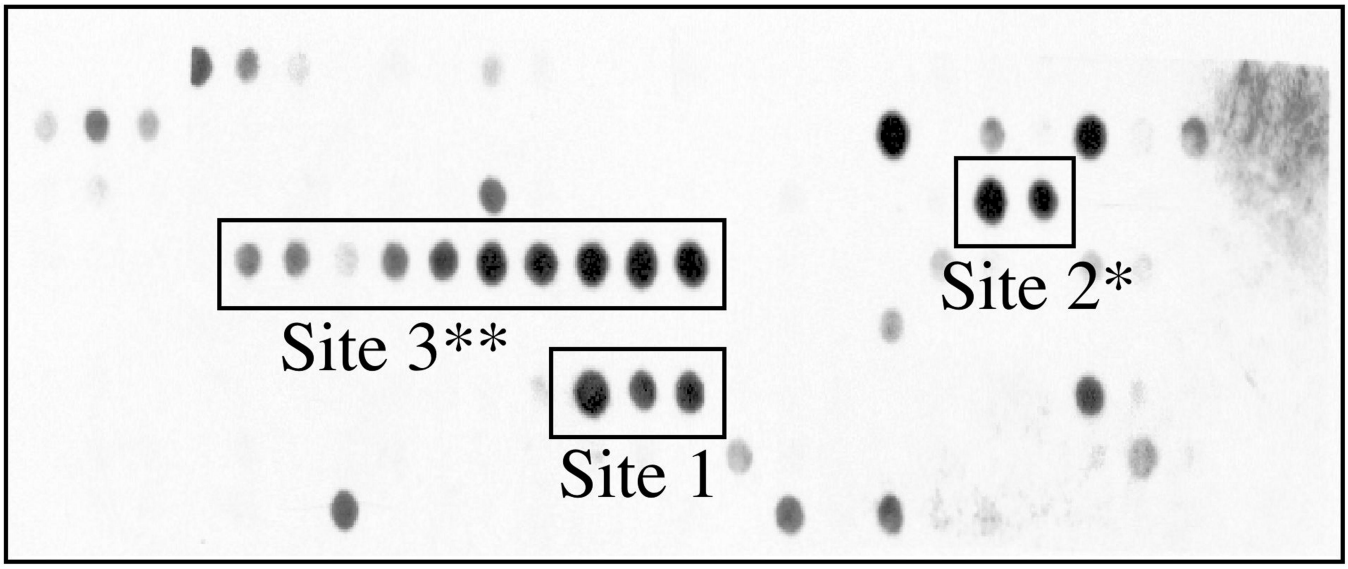


Figure 2

A



* Site 1: 257 - KILRLIDWGLAEFYHP - 272



* Site 2: 131 - RKIGRGKFSEVFEG - 144

** Site 3: 154 - VIKILKPVKKKKIKREIKILQNL - 176

B

Protein	Sequence homology
SV 40 Small t	121-KHENRKLYRKD----------131
Human CK2α	165-DHEHRKLRLIDWGLAEFYHP-184
Bovin CK2α	165-DHEHRKLRLIDWGLAEFYHP-184
P. falciparum CK2α	169-DHENRQIRLIDWGLAEFYHP-188
T. parva Site 1 CK2α	257-DHEKKILRLIDWGLAEFYHP-272

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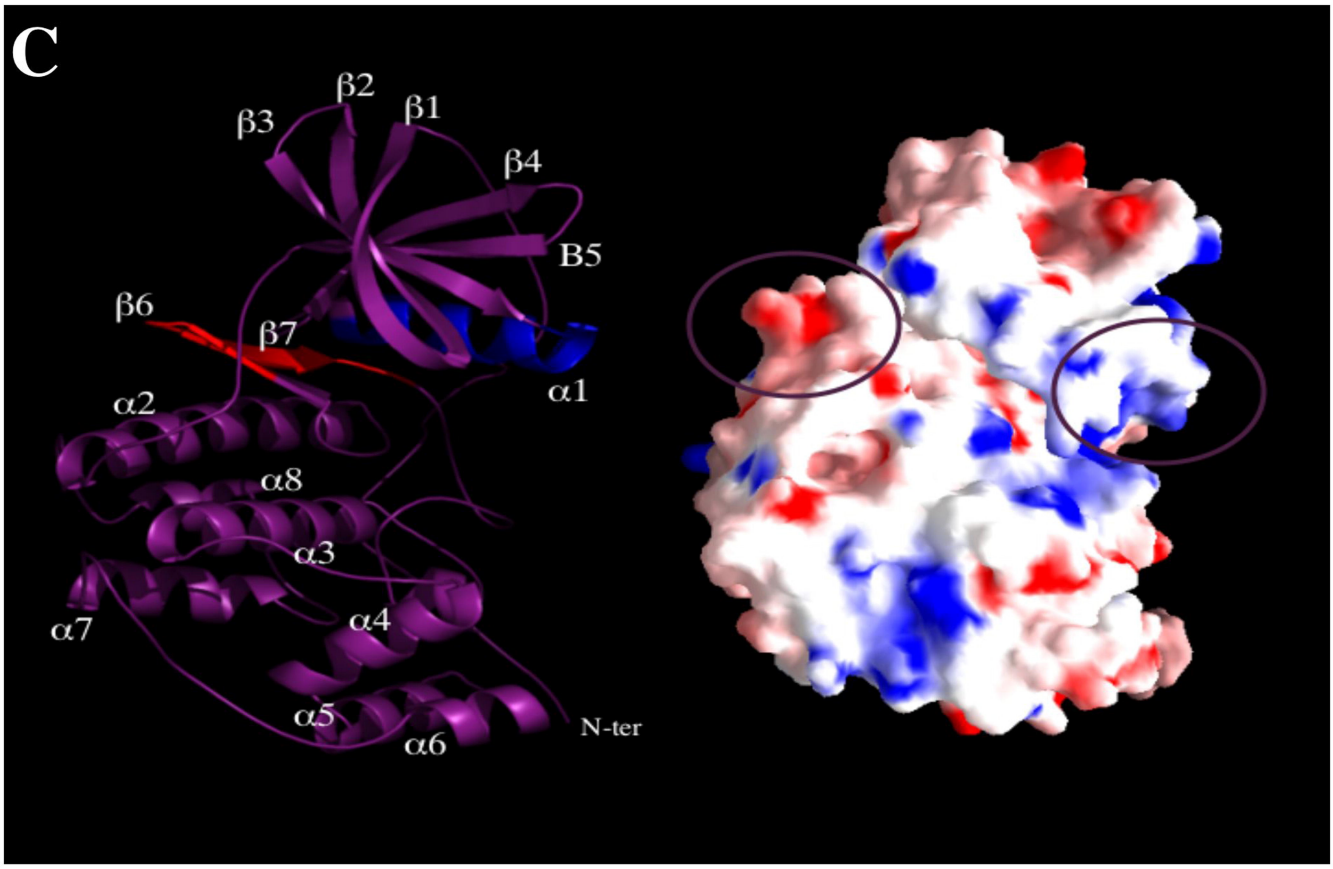
Figure 2



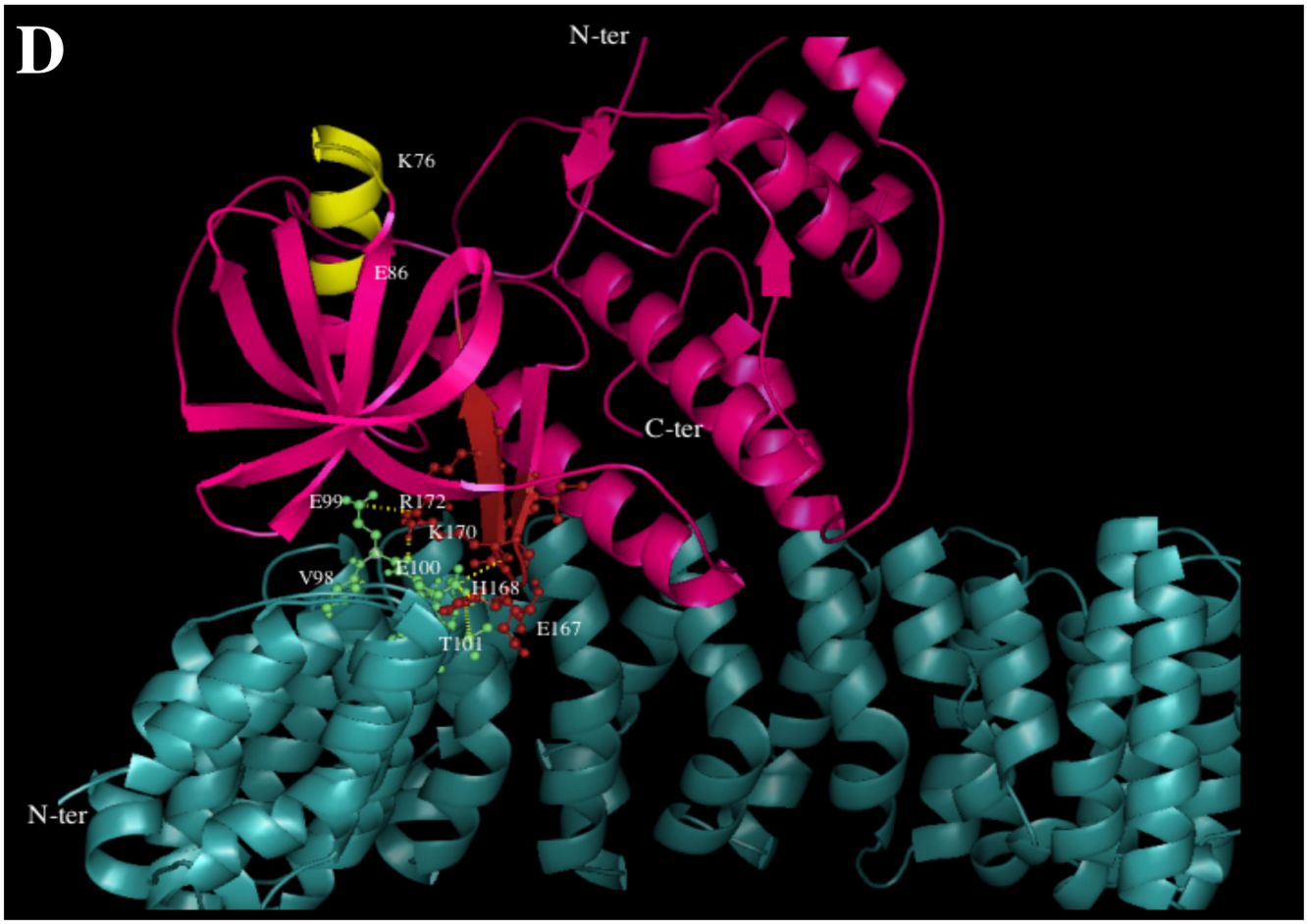


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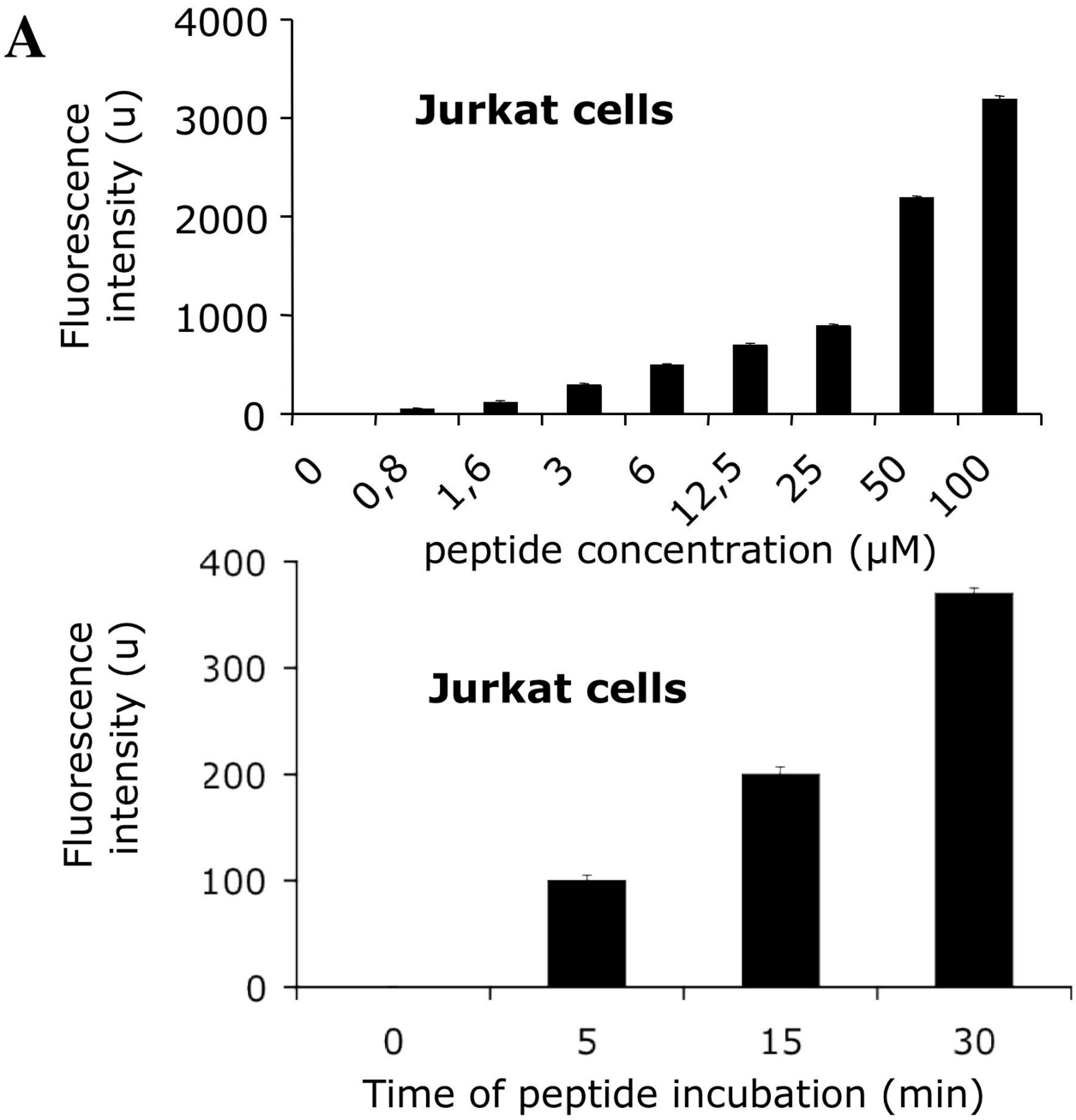
Figure 3



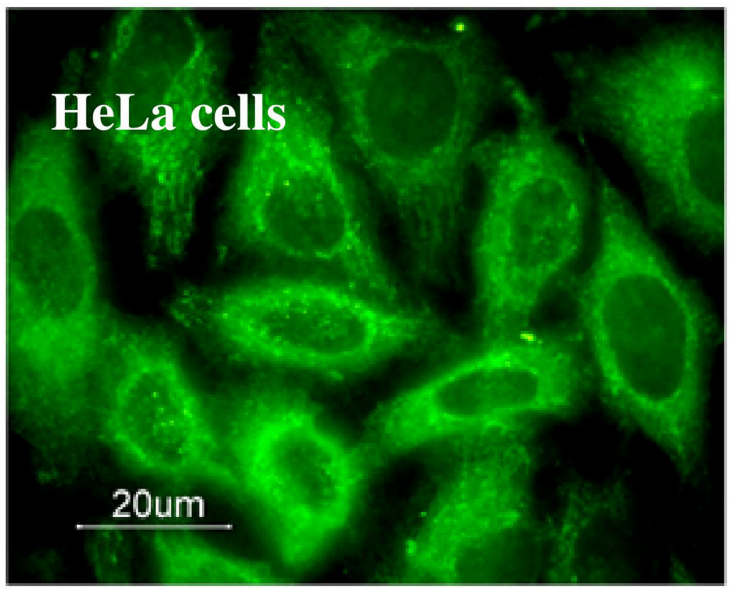


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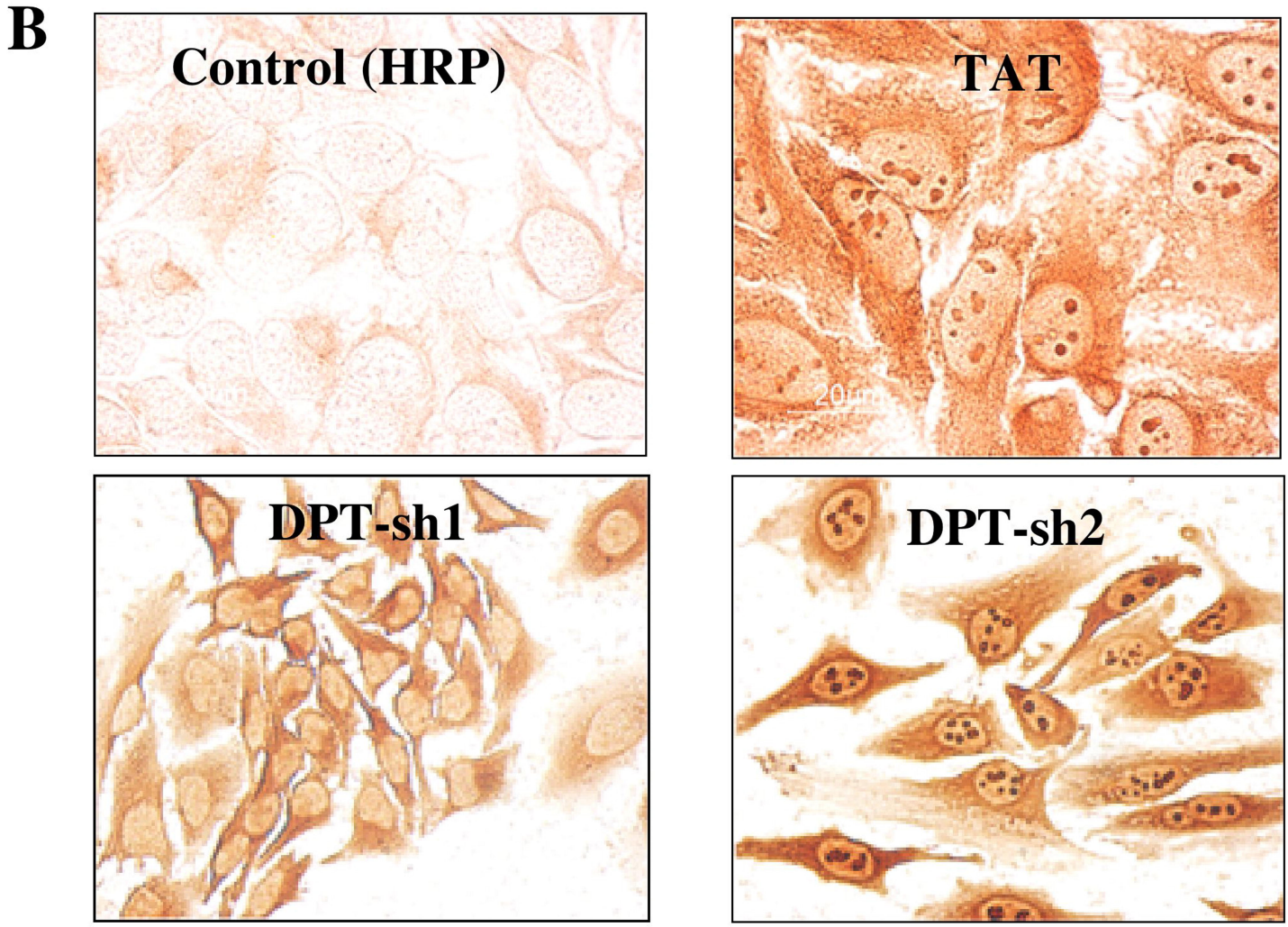
Figure 3



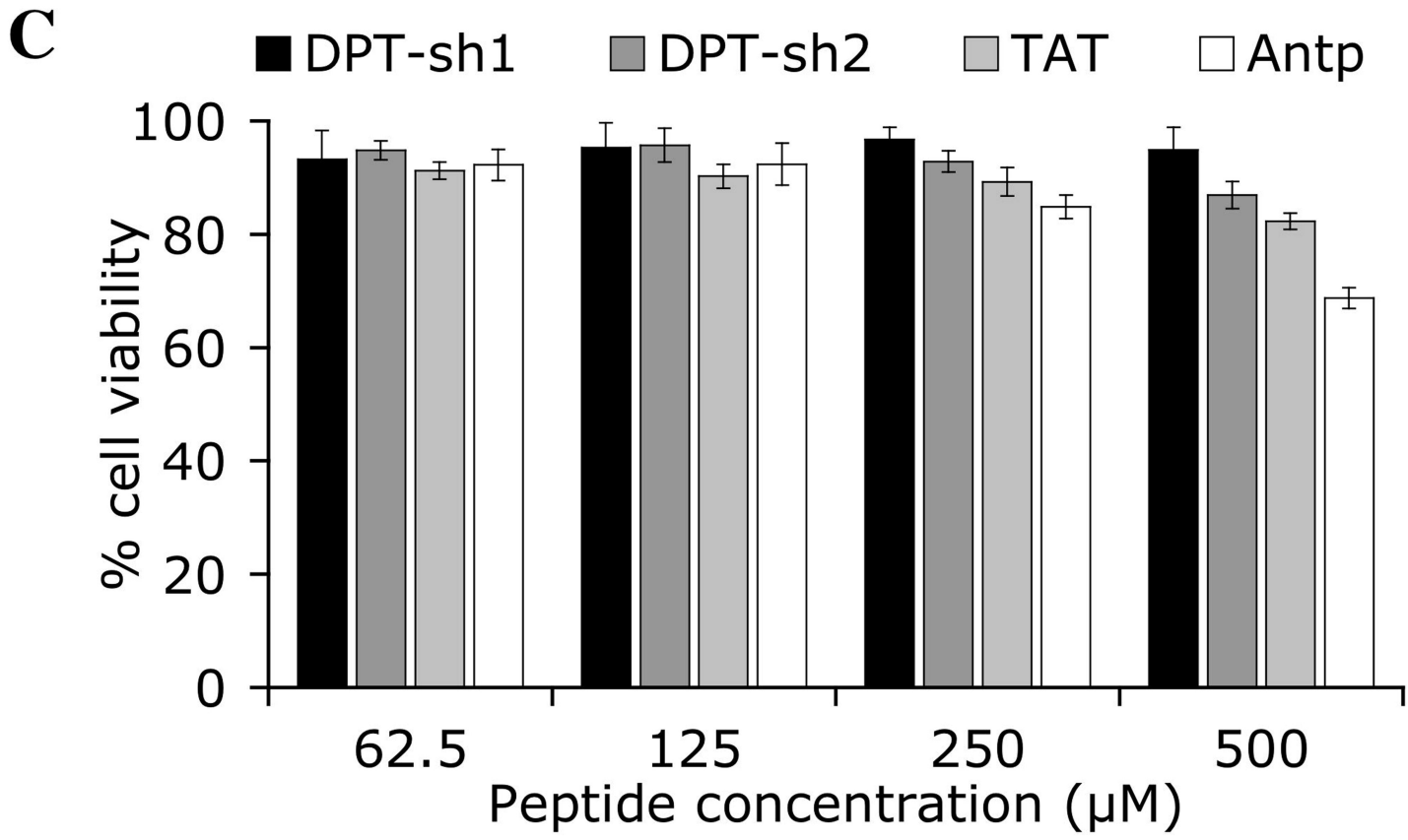


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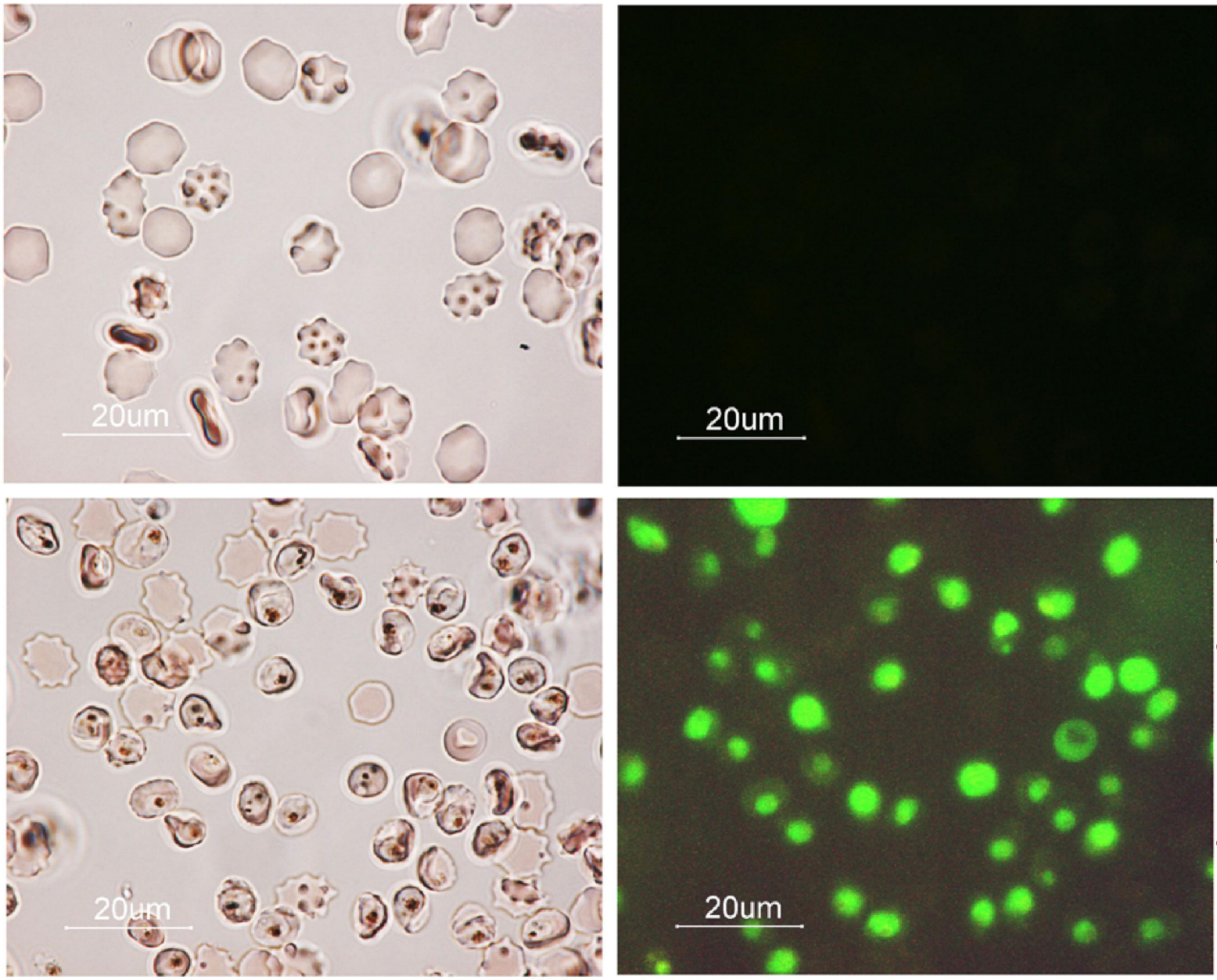
Figure 3

D



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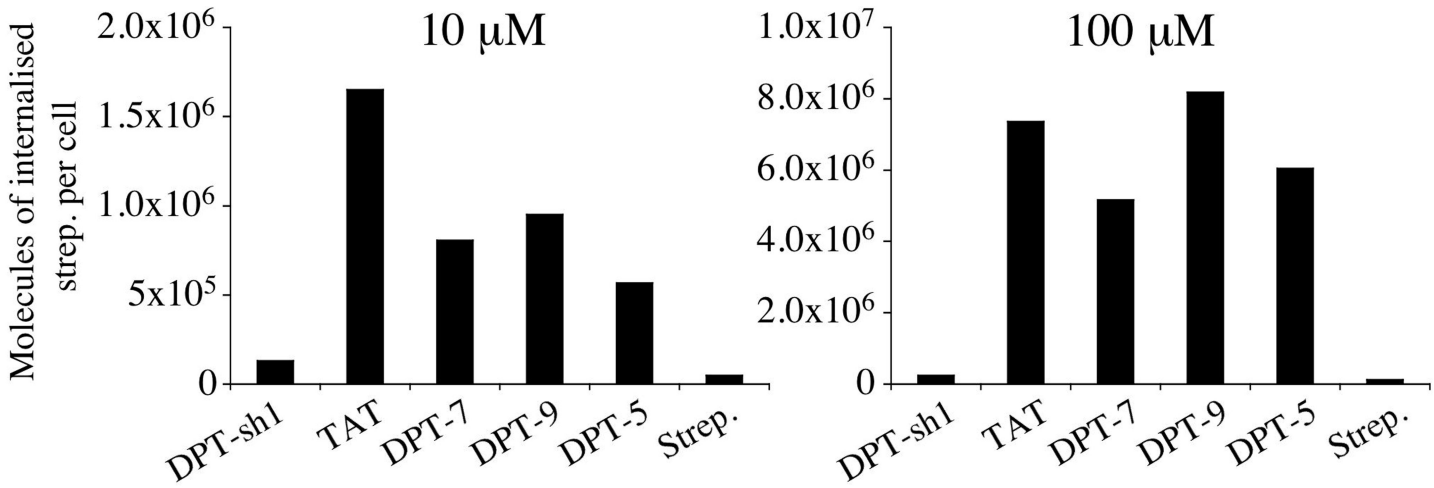

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Figure 4

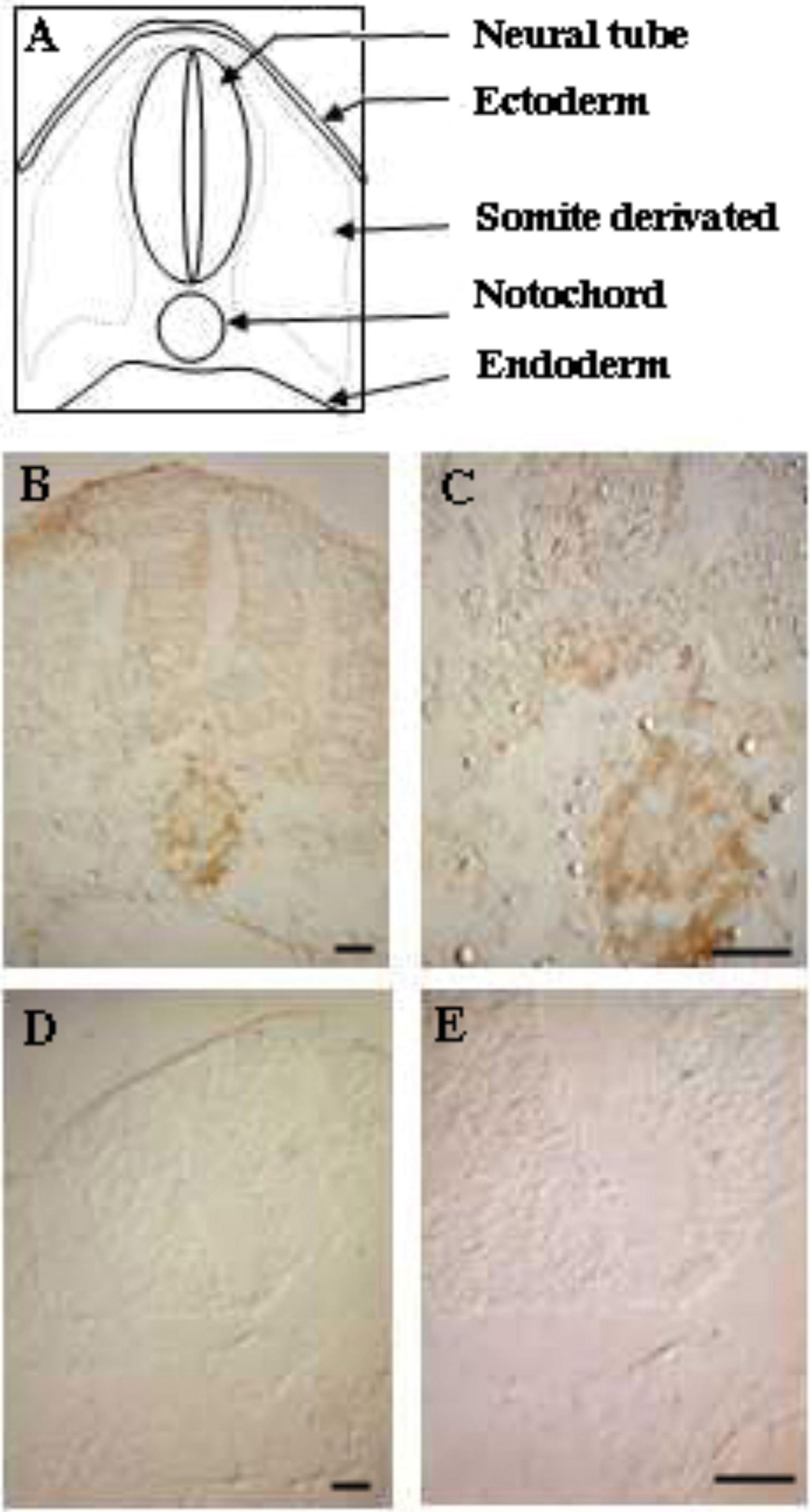

Figure 5

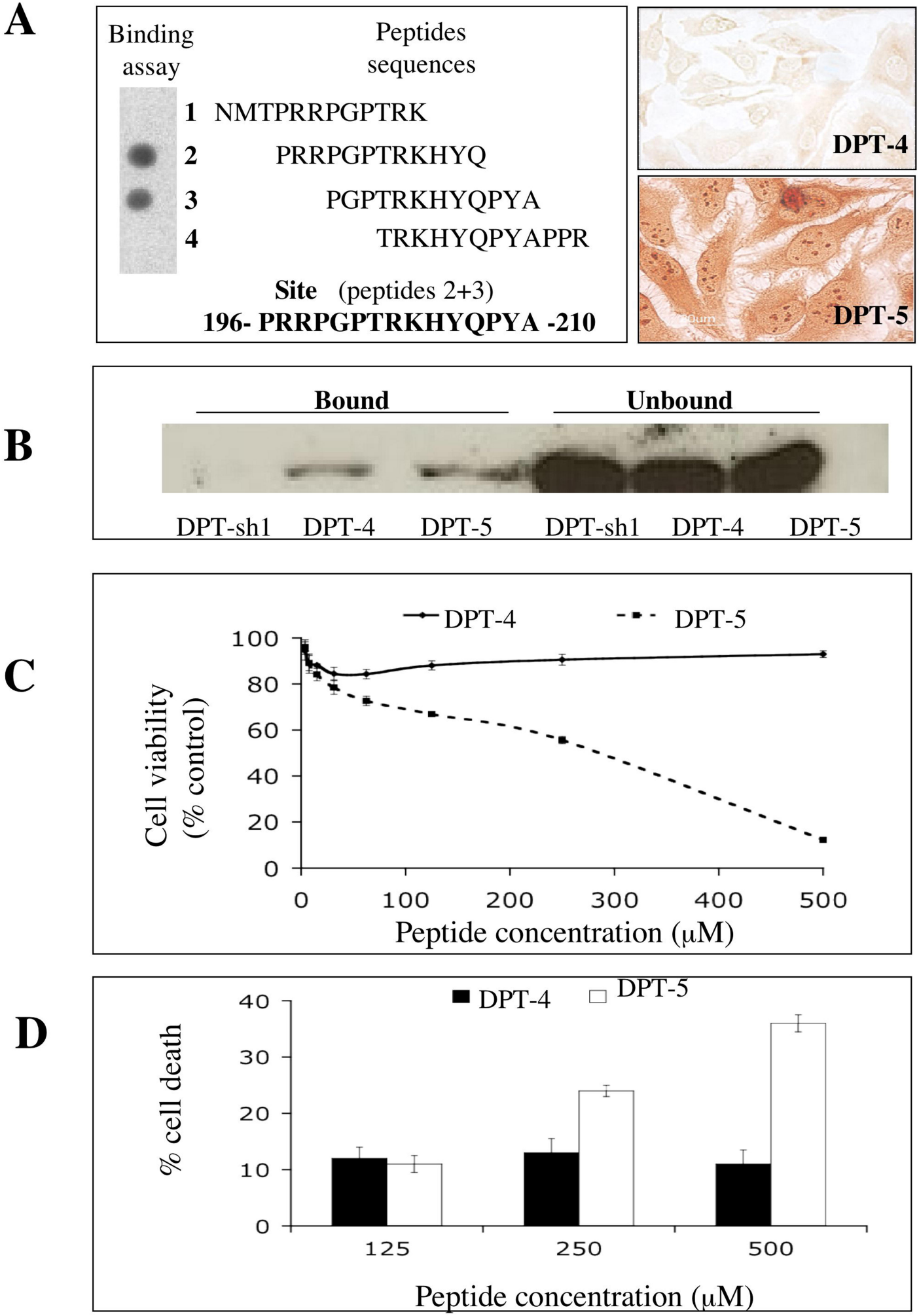

Figure 6