

Improving antitumor targeting via using PL3 homing peptide and cell-penetrating peptide

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Abstract

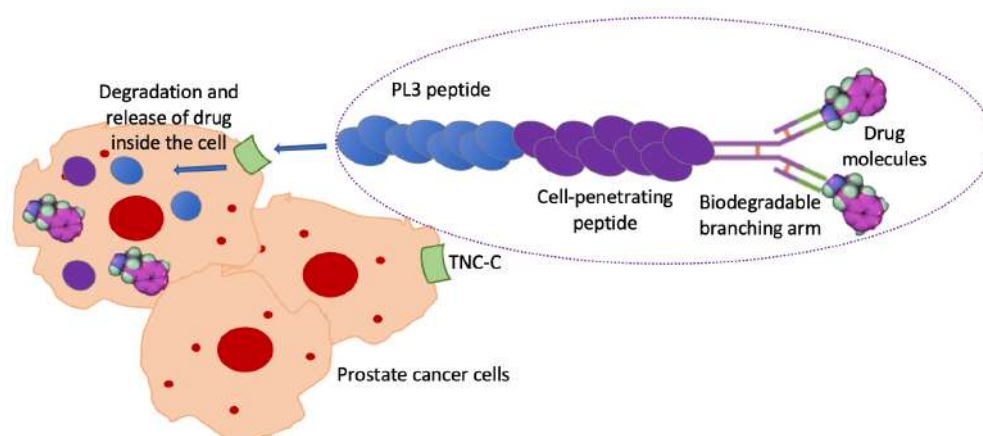
Introduction: Tumor-homing peptides have gained great attention as tools for the development of non-invasive and targeting drug delivery systems (DDS) to minimize drug systemic toxicity and enhance bioavailability. This study aims to improve antitumor targeting in prostate cancer via uploading a drug to a DDS comprised of a cell penetrating peptide decorated with a tumor-homing peptide, PL3.

Materials and Methods: The DDS was constructed via solid-phase peptide synthesis and then characterized via mass spectrum and high performance liquid chromatography. A cell viability assessment to evaluate its cytotoxicity on both tumor (prostate cancer cells) and normal cells was conducted, while a confocal laser scanning microscope and flow-cytometer were employed to investigate internalization. To inspect the effectiveness of the drug-loaded DDS, a biochemical enzyme inhibition assay on the target enzyme dihydrofolate reductase (DHFR) was performed.

Results and Discussion: The findings supported the succeeded synthesis and loading of the drug into this carrier system and demonstrated its high efficacy in cytotoxic effect and inhibiting DHFR with considerable cellular uptake in prostate cancer cells.

Conclusion: The drug was delivered to the target prostate cancer cells by the PL3-functionalized DDS, limiting its localization to tumor cells rather than normal cells. Therefore, the study results highlighted the significance of the DDS in tumor therapy interventions.

Graphical abstract:



Keywords

cancer chemotherapy, cell-penetrating peptide, drug delivery system, prostate cancer, tumor-homing peptide

Introduction

Malignant tumor is one of the leading causes of death due to diseases. Despite the fact that many approaches are used to treat cancer, including surgery and radiation, chemotherapy is the method of choice used at all stages of cancer treatment (Tang et al. 2021). Being one of the most effective ways of treatment, traditional chemotherapy has a number of disadvantages and limitations, the most critical of which is the development of drug resistance, which limits the transport of the drug and reduces the accumulation inside the tumor due to the overexpression of “multidrug-resistant (MDR) proteins”, such as P-glycoprotein (P-gp) in tumor. In themselves, chemotherapy drugs have a relatively low selectivity for cancer cells (Feng and Zhao 2017; Kebebe et al. 2018). As a result, cytotoxic drugs, such as [doxorubicin](#), [paclitaxel](#), [gemcitabine](#), [methotrexate](#) and [vinorelbine](#), have moderate efficiency, and cancer progression is usually observed with continued treatment due to drug resistance. An attempt to overcome this problem by dose increasing is limited due to the toxic effects of drugs on healthy cells in many organs and tissues (Ghaz et al. 2015; Lian et al. 2021). One of the main approaches to increase efficiency of chemotherapy for malignant tumors is the development of special drug delivery systems (DDSs), which also allows the therapeutic agent to reach the tumor cell selectively. Using these approaches, it is possible to reduce the dosage of the drug and its side effects, in relation to non-tumor tissues, retaining or even increasing chemotherapeutic activity. Nanomaterials and nanotechnologies have a potential impact in the DDSs development through increasing the circulation time of the drug, decreasing in nonspecific absorption with an increase in accumulation in the tumor tissue, as well as

stimulating endocytic uptake by tumor cells (Madamsetty et al. 2019). In recent decades, different types of carrier systems have been developed, including micellar nanoparticles, liposomes, polymers, and dendrimers. All drug carriers should be highly cell membrane permeable, capable of delivering pharmaceuticals without compromising their pharmacological action and be non-toxic. Encapsulation of drug molecules in DDS can improve their bioavailability, distribution, and can also improve internalization into the target cell. In the development of anti-cancer therapies designing, conjugates are used, where the DDS is linked to the drug through a biodegradable linker (Dutta et al. 2021; Alqosaibi 2022; Gao et al. 2022).

Until recently transport of hydrophilic macromolecules into living cell without destroying the membranes seemed impossible. At the same time, the delivery of biologically active macromolecules inside the cell opens wide opportunities for manipulating biological agents. Therefore, the discovery of peptides capable of penetrating the cell without destruction of membrane proteins and capable of carrying biomaterials effectively via intracellular transport initiates a new stage in the development of biomedicine and pharmaceutical industry. Cell penetrating peptides (CPPs), the peptides that can enter the cell, are usually isolated from the proteins of various organism viruses (HIV-1, herpes, influenza) and the spine (cayman). Fragment-immunodeficiency virus capsid protein volume HIV-1 is a penetrating peptide TAT (Richard et al. 2003; Ramsey and Flynn 2015; Guidotti et al. 2017), and herpes simplex virus- peptide VP22. The length of these cationic peptides ranges from 11 to 30 amino acids. An analysis of amino acid sequences revealed no homology; however, it was observed that they almost always composed of several molecules of

arginine, which have internalizing property (Richard et al. 2003; Regberg et al. 2012). The mechanism of transport of penetrating peptide through the cell membrane is not clear yet. However, it is proposed that CPP translocation proceeds through direct plasma membrane penetration (energetically independent) and endocytic pathways (energetically dependent) (Bolhassani et al. 2017; Guidotti et al. 2017). CPP, such as (TAT and VP22), are able to penetrate intracellular membranes and accumulate inside the cell, overcoming the resistance problem. Previous experiments have proven that CPP penetrate with equal efficiency into cells of different types. It has been documented that these peptides can transport into cells the covalently bound cargo, without subjecting for hydrolysis by lysosomal enzymes. Utilizing CPP as DDS allows detached drug molecules to be released solely after internalization in a method that can avoid Pgp-mediated efflux so drug is no longer a substrate for P-gp effluxes (Dubikovskaya et al. 2008; Regberg et al. 2012).

Development of a physiologically active delivery system, capable of easily penetrating through cellular membranes and targeting tumor cells, elicits new perspectives in cancer therapy, such as prostate cancer.

Prostate cancer is a bad prognostic metastatic disease and rated the third-leading cause of cancer-related deaths in males, despite the fact that there are numerous local therapeutic options. The standard therapy for metastatic prostate cancer is deprivation of androgen. Although survival has improved to some extent with using of new medications, such as cabazitaxel, abiraterone and enzalutamide, systemic adverse effects frequently necessitate discontinuing the treatment. Selective therapy techniques are desperately required to cope with the drawbacks of current contemporary therapies via targeting malignant tissue which minimizing adverse effects on intact tissues (Litwin and Tan 2017; Wada et al. 2019). For these reasons, a targeting ligand can be employed within the DDS to address the delivery of drug to the selective tissue.

Homing or targeting peptides have been identified with high interests for the developing less toxic medicines for some diseases, which are selectively able to transport a variety of compounds. These homing peptides are mostly short with 3 to 12 amino acids and have extremely high binding affinity and targeting selectivity. Using these targeting peptides in conjugation with antitumor drugs effectively promotes higher tumor regression when compared to free drug use (Yang et al. 2008; Kebebe et al. 2018; Zhao et al. 2018; Wada et al. 2019).

In this work, the homing peptide, PL3, which conjugates with the tenascin-C (TNC-C), which is increased in cancerous cells, is selected as prostate cancer targeting agent. This PL3 peptide was discovered by “in vitro biopanning on recombinant TNC-C”, it is of amino acid sequence: AGRGRLVR. In addition to TNC-C, PL3 interacts with the “cell- and tissue-penetration receptor neuropilin-1 (NRP-1)” through its C-end Rule (CendR) motif. Functionalization of metallic silver nanoparticles (AgNPs) and iron oxide nanoworms (NWs) with PL3 peptide improved systemic nanoparticles’ affinity for prostate cancer and glioblastoma xenograft lesions in mice. Studies have revealed an accumulation of PL3-coated nanoparticles in NRP-1 and TNC-C positive

regions of tumor tissue (Lingasamy et al. 2020; Ayo and Laakkonen 2021).

In order to potentiate the delivery of anticancer drugs to the target cells, PL3 (the targeting agent) was coupled to a CPP, TAT, to construct a DDS able to transport its cargo into prostate cancer cells selectively. **Methotrexate (MTX)** was used as a sample anticancer drug to be loaded covalently to the designed DDS. **MTX** is an antifolate antimetabolite which prevents the enzyme dihydrofolate reductase from converting dihydrofolate into tetrahydrofolate, the active form of folic acid. The synthesis of the nucleotides in both DNA and RNA depends on tetrahydrofolate. This mechanism is considered cytotoxic so being utilized in the treatment of cancer (Lindgren et al. 2006; Hannoodde and Mittal 2022). Long-term use of **MTX** is linked to a number of undesirable side effects, including suppression of bone marrow, stomatitis, ulceration of mucosa, liver cirrhosis and the propensity for cancer cells to develop resistance against drug action (Garg et al. 2016).

To our knowledge, however, the present study was the first to demonstrate enhanced **MTX** delivery employing a DDS made of TAT functionalized with PL3, providing an improvement against drug resistance and lack of selectivity with a substantial drug uploading capacity.

Material and Methods

Synthesis of targeting DDS

The synthesis by utilizing a “solid-phase peptide synthesis” (SPPS) and microwave technology (Biotag., UK) was first started on tentagel resin with PL3 peptide (AGRGRLVR), then coupled to amino acids of TAT peptide (RKKRRQRRR) with an extra lysine residue that provides a branching spacer function to create the carrier system (P-T). To synthesize the P-T-(MTX)₂ conjugate (chemical formula: C₁₃₅H₂₂₄N₆₅O₄₇ with MW 3509 Da), two drug molecules were conjugated by amide linkages at the NH₂ terminus of lysine. In the assembly, 0.4 mmol of Lysine (Fmoc-lys(Fmoc)-OH) and relative amino acids of peptides were used. Resin and other amino acids were obtained from Novabiochem Inc. (UK) and **MTX** from Sigma Aldrich (UK). The synthesis was carried out in accordance with the previously described procedure (Al-Azzawi et al. 2018; Meikle et al. 2011), which included coupling and deprotection of the amino acids as well as cleavage from the resin (Fig. 1).

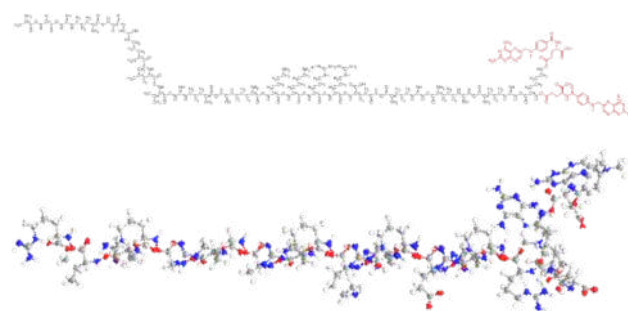


Figure 1. Structure of P-T-(MTX). In the up is the 2D one showing 2 attached **MTX** molecules in the red color. In the bottom is the 3D structure.

Drug molecules were subsequently coupled to each NH_2 terminus in a ratio of 1:1. Employing Zebaspin desalting column, and “high performance liquid chromatography” (HPLC) (Agilent Tech., UK), the final molecule was purified.

Characterization of the synthesized DDS

Utilizing mass spectrometry (Bruker, UK), the produced P-T-(MTX)2 conjugate was characterized. The sample is typically ionized when using an electrospray/ionization technique to produce multiple-charged ions with masses (m/z) that are labeled as $(\text{MW}+n\text{H})/n+$, where H is the proton's mass.

Via HPLC technique, the produced compound was also characterized and refined. A C18 column (150x4.6 mm) was used for the study, and the mobile phase solvents were water and acetonitrile, which were run over the course of 20 minutes in a gradient ratio of 75:25 to 25:75. The technique mode was reverse-phase chromatography using a hydrophobic column, and the chromatographer used UV-Vis detection.

Cell culture studies

Malignant prostate cancer cells, PC-3 cells (ATCC, USA,) were grown in RPMI-1640 media including 1g/mL fetal bovine serum (FBS), streptomycin and penicillin (100 IU/mL) (Gibco, UK) (Kamalidehghan et al. 2018). For experimental purposes, cells in the exponential growth phase that were 70%–80% confluent were grown in a humidified environment at 37°C using a 5% CO_2 incubator.

Non-cancerous cells, HUVECs, were grown in F-12 K media supplemented with 0.01 % of heparin and 0.05 mg/mL of ECGS “endothelial cell growth supplement”, in addition to 0.1 mL/mL of FBS in accordance with the instructions of the supplier.

For in vitro cytotoxicity assays, confluent cells were employed in the MTT experiments, and a range of concentrations (0.25 μM to 15 μM) of free drug, drug-loaded P-T, and un loaded P-T were used. The MTT assay was used to estimate the number of viable cells in multi-well plates following 24 hours incubation (Mosmann 1983). The absorbance at 540 nm was measured by spectrophotometer, and the obtained values were represented as a percentage of the control.

Biochemical investigation for the enzymatic inhibition

ELISA kit of human DHFR (Biorbyt Ltd., UK) was hired to assess the effectiveness of the loaded drug to suppress its target enzyme. PC-3 malignant cells were treated with 10 μM unbound MTX and MTX-loaded P-T, in addition to include control wells of untreated cells. The cells were lysed, centrifuged and examined spectrophotometrically at 450 nm. Utilizing a calibration curve drawn from sequential dilutions of standard solution, the amounts of active DHFR were determined (according to the supplier sheet).

Cellular uptake studies

Carrier system was labeled with “fluorescein-5-isothiocyanate (FITC)” (Thermo Fisher Sc., UK) to evaluate its cellular uptake. FITC was used in a dark coupling procedure to label the DDS via attaching to the

terminal amino group in a ratio of 2:1. At confluence, the wells were incubated for 30 minutes with FITC-tagged solution. Following 3.7% v/v formalin fixation, the cells were examined using “confocal laser scanning microscopy” (CLSM) (Leica TCS, UK).

To evaluate internalization flowcytometrically, the BDC6-sampler (Bioscience, UK) was used on treated and control cells; this examination was done using the FL1-H channel.

Statistical analysis

The experiments are reported as mean \pm standard deviation (SD) of a number of independent experiments (n). The obtained data were analyzed using one-way ANOVA and Tukey's tests. Significance is statistically defined if P-value is less than 0.05.

Results and Discussion

Characterization of the carrier system

An analysis of produced molecule by mass spectra detected various peaks representing its ions abundance as a result of the fragmentation according to mass/charge ratio (m/z). With a theoretical molecular weight of 3509 Dalton, the data confirmed the successful assembly and loading of MTX to the carrier system. The result was supported by detection of a major peak of a double charged ion (positive) and other related ions as shown in Fig. 2.

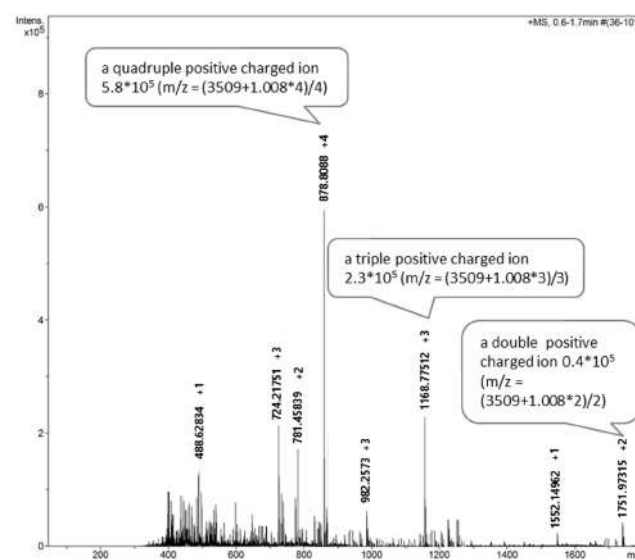


Figure 2. Mass spectrum of P-T-(MTX) showing the related ions.

To produce a pure final product and remove any undesirable byproducts, many samples were isolated throughout HPLC analysis. After being freeze-dried, the samples were analyzed, and the purity was confirmed by appearance of the final, clear and uncontaminated peak at 14.6 minutes (Fig. 3).

In this study, a short TAT peptide sequence was selected as the CPP to improve MTX penetration utilizing prostate cancer cells. With the aid of functionalization

with the tumor-homing peptide PL3, this DDS can target the delivery of **MTX** to malignant prostate tissues only. Results from mass spectra and HPLC showed that the SPPS approach was successfully used to synthesize the DDS and to load **MTX**. Numerous peptides were produced with a considerable purity, precision and reproducibility, using microwave-based SPPS (Merrifield 1965; Rodríguez, Suarez, and Albericio 2010; Al-Azzawi et al. 2019).

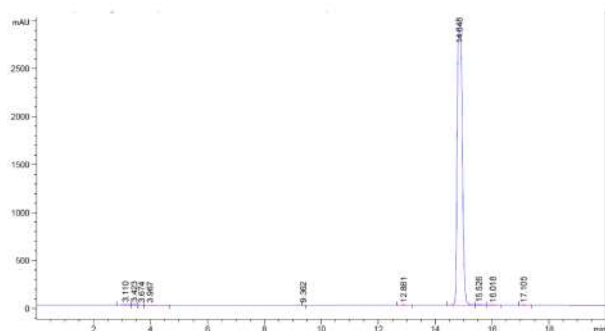


Figure 3. HPLC analysis of P-T-(MTX)2.

Cytotoxicity assays

In each tumor cells (PC-3) and non-tumor cells (HUVEC), the cytotoxicity of the unloaded and MTX-loaded P-T delivery system, and the free **MTX** was assessed. A significant inhibition of growth ($P < 0.05$) was found with P-T-(MTX)2 treatment in comparison to numbers of the unbound **MTX** treatment for more than 1 μM concentration; which may be because of the intracellular accumulation of delivery system due to impact of CPP internalization (Dissanayake et al. 2017; Ramsey and Flynn 2015). The toxic effects on malignant PC-3 cells were enhanced by augmenting the concentrations of each free drug and drug-loaded into this DDS.

Even at the higher dose employed in the assay (15 μM), unloaded P-T did not exhibit any cytotoxicity in either cells confirming the safety of this carrier to be used for various purposes.

When compared to the results obtained from the free drug in 10–15 μM , the cytotoxic response to P-T-(MTX)2 was significantly ($P < 0.05$) lower with regards to effects on the HUVEC cell line. In addition, the statistical analysis of the drug-loaded DDS molecule revealed that the viable number of the treated malignant cells was significantly ($P < 0.05$) lower than that of the treated HUVEC (healthy cells) of the correlated concentrations, indicating low cellular penetration of the MTX-laden carrier system into healthy cells. The reason can be due to the delivery system's functionalization with PL3, which produces targeted penetration that only detects tumor tissue (Fig. 4). Our results are in agreement with the previous findings when PL3 was used to target the delivery of metallic silver nanoparticles and iron oxide nanoworms to the prostate cancer in mice (Lingasamy et al. 2020; Kondo et al. 2021).

PL3 homing peptide interacts with the TNC-C, which is increased in cancerous tissues and with NRP-1 via its CendR motif. In clinical tumor samples, it was documented that PL3-coated nanoparticles accumulated

in TNC-C and NRP-1-positive regions, indicating a potential translational relevance. This PL3 peptide of 8-amino acid has the benefit of being smaller, which makes it less potential as immunogenic than a chimeric 19-mer peptide (another tumor homing peptide) (Lingasamy et al. 2020). Moreover, it is easier to be created and developed for different therapeutic applications than others. This peptide has also the CendR motif exposed at the C-terminus so eliminating the necessity for on-site proteolytic activation that is required for a number of other tumor homing peptides.

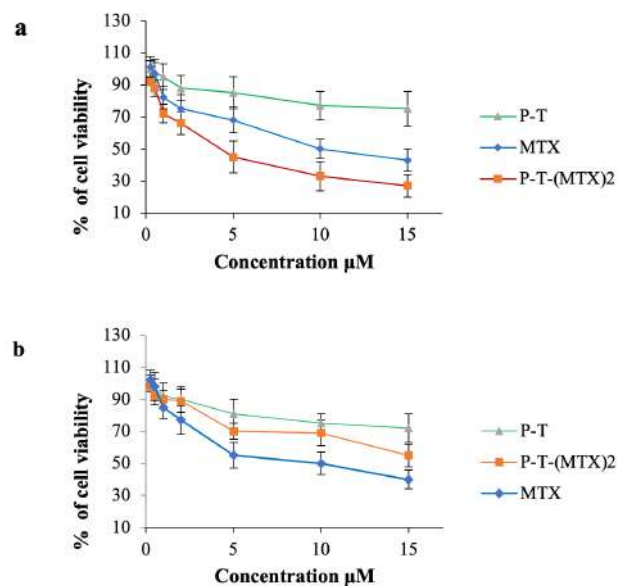


Figure 4. MTT results of treated cells with unbound **MTX**, MTX-bound P-T and free P-T. **A.** PC-3, **B.** HUVEC. The data represent mean \pm SD ($n = 6$).

It was determined that a phage clone exhibiting PL3 with C-terminus RLVR residue surpassed other phage clones. It demonstrated stronger TNC-C binding in the cell-free experiment during investigations employing various phage clones. It has also been documented that extravasation transport across malignant cells are mediated by an interaction between the C-terminal RLVR of PL3 and the b1 domain of NRP-1 (Teesalu et al. 2009; Lingasamy et al. 2020). Hence, this PL3 peptide might have implications as a tumor targeting agent for the preferential delivery of medicines or imaging agents due to its tumor-targeting characteristics and binding to solid tumor parts.

Biochemical investigation for enzymatic inhibition

The inhibition of activity of DHFR, the enzyme target of **MTX**, was measured biochemically using the “ELISA technique” to further support the results mentioned above and to assess the effectiveness of the antitumor agent after loading on the constructed delivery system and the impact of the covalent bonding on therapeutic action of drug.

When malignant cell culture was incubated with P-T-(MTX)2 conjugate, the enzyme activity inhibition (98 ± 23) was significantly increased ($P < 0.0001$) in comparison with the effect of unbound **MTX** (145 ± 38), indicating that **MTX** potentially maintained its pharmaceutical action after loading on this DDS and even displayed greater effectiveness (Table 1).

Table 1. Estimation of DHFR enzyme activity.

	Replica- tions (n)	Levels of DHFR pg/ mL (Mean ± SD)	p Value to control	p Value to MTX
Control	6	380±43		
MTX	6	38±145	<i>p</i> <0.0001	
P-T- (MTX)2	6	23±98		

Note: DHFR – dihydrofolate reductase; MTX – Methotrexate; P-T-(MTX)2 – C₁₃₅H₂₂₄N₆₅O₄₇ with MW 3509 Da.

This substantial response of MTX–conjugated P-T could be elucidated by the internalization of peptide-based DDS and liberation of MTX intracellularly owing to amide linkage degradation, taking in consideration the loading capacity of the delivery system following the introduction of biodegradable branching lysine arm.

A variety of impermeable payloads may now be delivered into cells using peptide-based delivery systems (Soler et al. 2015; Joncour and Laakkonen 2018; Al-azzawi and Masheta 2019; Al-azzawi et al. 2020). It has been demonstrated that CPP can improve the cytotoxic effect of anticancer medications by enhancing their bioavailability (Shin et al. 2014; Bolhassani et al. 2017). It is generally accepted that CPP internalization takes place when their highly positive charges interact with the negative charges of the cellular membrane. This interaction leads to endocytosis, which then transports CPPs to the cellular lysosomal compartment for degradation. The peptide bond is anticipated to be broken down by lysosomal proteases, particularly cathepsin B, releasing the active moiety (free drug) (Diop et al. 2011; Soler et al. 2015). Incorporating degradable subunits during assembly causes the peptides to completely dissociate, releasing all of their components, including the drug molecules due to bond cleavage by proteases (Xu et al. 2014). This is substantiated by earlier research that has shown full activity of drug owing to linkage dissociation when MTX was coupled via amide linkage to a poly-lysine polymer (Silverman and Holladay 2014). The utilization of lysine monomer as a spacer during construction that covalently binds to the bioactive agents with assured efficacy as indicated by this study finding may hold a considerable promise for further applications.

Cellular uptake studies

Imaging by CLSM revealed a distinct intracellular localization and deposition of fluorescence in PC-3 cell cancer, which referred to the uptake of DDS (Fig. 5). The DDS’s cellular uptake was also investigated using flow cytometry. When PC-3 was incubated with the labelled carrier molecule, the fluorescence intensity was remarkable (92%), 3.8 times higher than that of non-cancerous HUVEC (24%) (Fig. 6).

Notably, the functionalization with PL3 boosted the localisation of DDS in cancerous cells rather than healthy ones, highlighting its selectivity and limited the uptake to cancerous cells only.

Therefore the designed molecule, which incorporates TAT and PL3, exhibits promising internalization capabilities for utilization in cancer targeting and drug delivery systems. According to earlier studies, the abundance and spatial configuration of the guanidinium moities in the Tat 49-57 (RKKRRQRRR) molecule and its high content of arginine is responsible for the effective cellular uptake (Vargas et al. 2014). It is proposed that concurrent adaptive translocation and endocytosis processes could enhance the internalization (Lee et al. 2008). Furthermore, several studies have indicated that internalization of a variety of cargoes has been facilitated by guanidinium-rich CPPs (Wender et al. 2000; Cooley et al. 2012; Stanzl et al. 2013). For instance, in guanidinium-rich CPP when conjugated to taxol, its cellular transport has amplified and tumor cells' resistance has overcome (Vargas et al. 2014). Previous studies revealed that chitosan-TAT-decorated with doxorubicin complex had improved tumor uptake and addressed transport of a loaded medication (Ghaz et al. 2015; Shin et al. 2014). Furthermore, an investigation has revealed that TAT-Lip-DNA complex greatly improved uptake and decreased cytotoxicity (Torchilin et al. 2003). Moreover, a TTP-functionalised TAT has improved cellular uptake and targeting of MTX into breast cancer cells in a previous work (Al-azzawi and Masheta 2019).

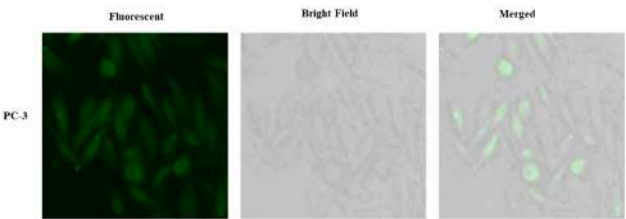


Figure 5. CLSM images exhibit high accumulation of contrast molecules internalized by the malignant cells (scale 50 µm).

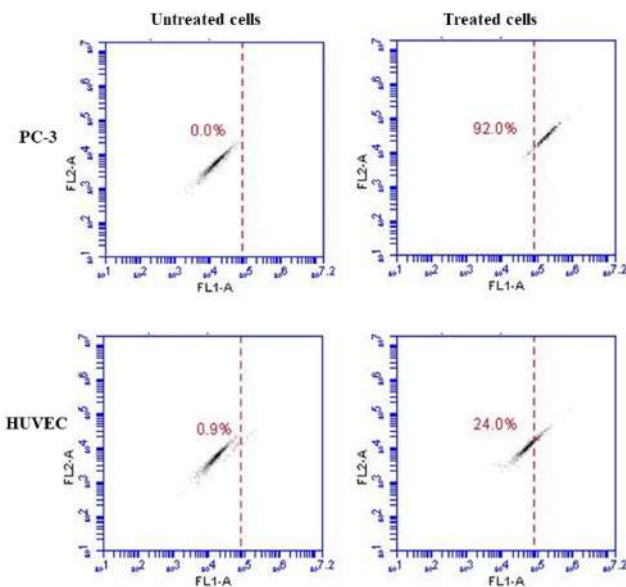


Figure 6. Flow cytometry’s uptake results show higher percentage by PC-3 than that by HUVEC cells.

Ultimately, the synergistic integration of the TTP with CPP enhanced both the internalization and prostate tumor targeting of MTX, which also can reduce the dose required for the therapeutic activity. Additionally, by delivering the chemotherapy only to the targeted tumor this could minimize interactions with healthy parts and in turn the unfavorable systemic side effects. As indicated above, the TTPs selectively interact with receptors that are overexpressed on tumor tissues, but they on their own are unable to enter tumor cells. Nevertheless, CPP can effectively be internalized by the cell but lack the targeting property. Therefore, combining TTP and PL3 with a biodegradable branching lysine arm is thought to be a potential conjugation that can substantially enhance capacity for drug permeability and prostate cancer targeting.

Therefore, future research to improve the distribution of various anticancer medications or diagnostic and imaging agents can make advantage of this DDS.

Conclusion

This study describes the impact of exploiting a DDS comprising TAT, a cell penetrating peptide and PL3 (an 8-amino acid homing peptide) which interacts with TNC-C and NRP-1 of the cancerous cells. PL3-functionalised TAT guided DDS to be accumulated in prostate cancer cells in vitro. This DDS can be beneficial for tumor

treatment, diagnosis and imaging and can act as a tumor-seeking for different antitumor cargoes and its influence in clinical practice could be more noticeable in the near future.

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Human and Animal Rights

“This article does not contain any studies with human or animal subjects performed by any of the authors. All institutional and national guidelines for the care and use of laboratory animals were followed”.

Conflict of Interest

The authors have declared that no competing interests exist.

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