Cell-penetrating doxorubicin released from Elastin-like polypeptide kills doxorubicin-resistant cancer cells *in vitro* study

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Abstract

Background: Elastin-like polypeptide (ELP) undergoes its characteristic of phase transitioning in response to ambient temperature. ELP therefore has been be used as a thermosensitive vector for the delivery of chemotherapy agents since it can be targeted to hyperthermic tumors. This novel strategy introduces unprecedented options for treating cancer, with fewer concerns about side effects. In this study, the ELP system was further modified with an enzyme-cleavable linker in order to release drugs within tumors. This system consists of ELP, a matrix metalloproteinase (MMP) substrate, a cell penetrating peptide (CPP), and 6-maleimidocaproyl amide derivative of doxorubicin (Dox). This construct may be initially targeted to the tumor by application of mild heat after administration. Within the hyperthermic tumor, then this construct is cleaved by MMP, releasing CPP-Dox, which can infiltrate tumor tissues and penetrate cell membranes.

Methods: We produced the construct in *E.coli* and examined its cleavage by MMP enzymes *in vitro*. Flow cytometry and confocal analysis were used to verify the facilitated uptake of the digested cell-penetrating Dox by breast cancer cells and Dox-resistant cells. Cytotoxicity tests further demonstrated improvements in bioavailability of cell-penetrating Dox following the enhanced cellular uptake of the cancer cells. Comparisons with the non-cleavable ELP counterpart were paralleled.

Results: This strategy shows up to a 4-fold increase in cell penetration and results in more death in breast cancer cells than the ELP-Dox. Even in doxorubicin-resistant cells (NCI/ADR and MES/ADR), ELP-released, cell-penetrating doxorubicin demonstrated better membrane penetration, leading to at least twice the killing of resistant cells than ELP-Dox and free Dox.

Conclusion: MMP-digested CPP-Dox shows better membrane penetration and induces more cancer cell death *in vitro*. This CPP-complexed Dox released from ELP kills even dox-resistant cells more efficiently than both free doxorubicin and non-cleaved ELP-CPP-Dox.

Background

It is acknowledged that current conventional chemotherapy is comprised of mostly cytotoxic drugs. These drugs have a strong anticancer efficacy, but cause collateral damage to non-tumor tissues. These unwanted side effects usually render a dose-limiting factor for chemotherapy and are a main reason for unsatisfactory prognosis of the therapy. Many efforts have been tried to resolve these problems, usually by attempting to raise the therapeutic index of the chemotherapy.

Curing cancer is certainly one of the greatest challenges of our time, and to confront it, our knowledge of cancer has been greatly growing in the last decades. In recent years, there has been a surge of new technologies aiming for cancer treatment such as molecular targeted therapies (i.e. anti-tyrosine kinase and anti-HER2) (1), immunotherapies such as cancer vaccines or anti-PD1 (2, 3), sophisticated radiation therapy (4) and advanced tumor targeting technologies such as nanoparticles and antibody drug conjugates. These technologies could make a big difference in the way we treat cancer, bringing us closer

to being able to ‘cure’ this disease. In particular, the nanosized drug delivery technologies have been significantly improved, and many of them are currently being used to solubilize the drugs, bypass immune-surveillance, sensitize current therapies and target tumor tissues (5, 6). A tumor targeting technology is to specifically deliver drugs to tumor tissue so that the concentration of drugs in tumor tissue will increase compared to the concentration in other normal tissues. This allows more opportunity for a drug to express its activity on tumor cells, resulting in selective death of cancer cells with tolerable side effects.

Elastin-like polypeptide (ELP), a thermo-responsive biopolymer, is a bio-polymeric carrier for targeted drug delivery. ELP, as a derivative from tropoelastin, consists of repeats of pentapeptides (valine, proline and glycine) (7, 8). The repeats of these hydrophobic amino acids permit ELP to have a unique re-arrangement of molecules responding to a surrounding temperature, which is a thermo-responsive phase transition. At low temperature, ELP remains as a monomer and soluble in solution; however, it co-acervates and precipitates in solution when ambient temperature rises above its phase transition temperature (9-11). This co-acervation can also be reversed decreasing the temperature of the ELP solution. Thus, this reversible phase transition of ELP is mainly under the control of surrounding temperatures, and ELP are highlighted as a controllable carrier for anticancer drugs in active tumor targeting strategies.

Additionally, ELP also exploits an ‘enhanced permeability and retention (EPR) effect’ and can progressively accumulate in tumor tissue due to abnormal histological structures of tumor. These unique properties render ELP itself a drug carrier that can exploit both the EPR effect and tumor targeting using the hyperthermic technique (12). Furthermore, ELP has been modified by the addition of cell penetrating peptides (CPP) to allow enhanced cellular uptake, improved penetration of physiological barriers like the blood-brain barrier, and preferential intracellular distribution such as in the cytoplasm or the nucleus (13, 14).

Many previous researchers have verified the potential of this polymer (15-18), and animal studies that include the use of local hyperthermia have demonstrated that ELP is able to deliver a sufficient amount of drugs to the tumor area showing significant tumor reduction efficacy. In this study, we further modified the ELP drug delivery system to release drugs in response to an enzyme which is abundant in tumor tissue. The suggested system is composed of ELP; a matrix metalloproteinase (MMP) substrate, mmpL; a cell penetrating peptide, CPP; and a 6-maleimidocaproyl amide derivative of doxorubicin, Dox (Figure 1A). In this study, we generated an MMP-responsive ELP drug delivery system that releases CPP-Dox, and we report the usefulness of this strategy to overcome Dox-resistance by investigating cellular uptake and anti-proliferation properties of the proposed system.

Methods

Design of construct and protein preparation

ELP has 150 repeats of VPGXG with guest residues (amino acid at position X) of Val, Gly, and Ala in a 5:3:2 ratio. ELP coding sequences were modified by the addition of the “Tat” cell penetrating peptide sequence (YGRKKRRQRRR), an MMP cleavable sequence (PLGALG) and three Gly-Gly-Cys residues to the C-terminus of ELP for the conjugation with Dox (Table 1). For an MMP uncleavable control, six Gly residues were used instead of the MMP cleavable sequence. All constructs were expressed in the *Escherichia coli* strain BLR(DE3) using pET 25b as an expression vector and were purified by repeated inverse transition cycling.

Conjugation of ELP constructs with doxorubicin or fluorescent probes

100 uM protein in PBS was reduced with 1 mM of tris-(2-carboxyethyl) phosphine (TCEP, Molecular probes) for 30 min at room temperature. Conjugation with 200 uM of the 6-maleimidocaproyl amide derivative of doxorubicin or tetramethylrhodamine-5-iodoacetamide dihydroiodide (Molecular probes, Eugene, OR) was followed by incubation at 4 °C overnight. Conjugated peptides were purified by inverse transition purification as described previously (9), and the concentration and the labeling efficiency were assessed by UV–visible spectrometry (UV-1600, Shimadzu). Concentrations of labeled ELP polypeptides were determined using the following equations:

See equations 1 and 2 in the supplementary files.

Cell culture

MDA-MB-231, MCF7, NCI-ADR, MES-SA, MES-SA-5Dx and SKBR3 cell lines were obtained from ATCC (Rockville, MD, USA). HT1080 was a generous gift from Dr. Michael Herbert of University of Mississippi Medical Center. All cell lines were grown and maintained at 37°C, 5% CO_2 in Dulbecco’s Modified Eagles Medium with 10% fetal bovine serum.

Cleavage assays

Recombinant-human pro-MMP-2 (Enzo life science) was activated with 2.5 mM 4-aminophenylmercuric acetate at 37°C for 2 h. Then 1ug of each ELP construct was incubated with the pre-activated MMP-2 (10 pmol) for 4 h in reaction buffer (50 mM Tris, 200 mM NaCl, 10 mM, $CaCl_2$, and 10 mM $ZnCl_2$, pH 7.5). After reactions were loaded and separated on an SDS-PAGE gel, each peptide’s cleavage pattern was confirmed by silver staining and by scanning the fluorescence of the gel with the IVIS Live Animal Imager (Caliper Lifesciences).

Flow cytometry analysis of cellular uptake

Cells were incubated with each treatment for 2 h at 37°C. Cells were rinsed with PBS and collected by trypsinization. Intracellular fluorescence was measured using a Gallios Flow Cytometer (Beckman Coulter) after trypan blue quenching as described previously (19). Forward versus side scatter gating was used to remove cell debris from the analysis, and the mean cellular fluorescence intensity was recorded.

The mean cellular fluorescence was corrected for differences in labeling efficiencies among polypeptides, and the results shown are an average of at least 3 experiments with bars representing SEM.

Cytotoxicity test

Cells were plated in a 96-well plate and treated with a range of concentrations of each treatment for 24 h at 37°C. After further incubation with fresh media for 48h, cell viability was assessed using the MTT assay (Sigma). Briefly, a 0.5 mg/ml solution of thiazolyl blue tetrazolium bromide dissolved in PBS was added to each well and the plates incubated for 4 h at 37°C. Formazan formed by mitochondrial reduction was dissolved in 100 uL of DMSO and its absorbance was read at 570 nm. Survival rate of each group was calculated in comparison to a vehicle-treated control group.

Confocal microscopy

Cells (10^3 cells/chamber) were plated in 2-well Lab-Tek CC2 chamber slides (Nunc). After 24 h incubation at 37°C, the cells were treated with each treatment for two hours at 37°C. The cells were washed three times with PBS, fixed with cold 4% paraformaldehyde and stained with DAPI (Molecular probe) for 10 min at RT to visualize the nucleus. Distribution of each molecule was investigated by laser scanning confocal microscopy with a 60x oil immersion objective (Leica).

Results

Incubation of ELP-mmpL-CPP with MMP-2 produces cleaved CPP

Figure 2A depicts how ELP-mmpL-CPP would be cleaved by MMP, producing ELP (60KDa) and cleaved CPP (Tat peptide, 3KDa), while the other construct, ELP-CPP-Dox (63KDa), will not be digested by MMP. This hypothesis was verified by the following experiments. MMP-2 was used for the digestion in this experiment since MMP-2 is involved in the degradation of extracellular matrices in tumors and is overexpressed in most tumors compared with normal tissues (20). After incubation of each rhodamine (rho)-labeled construct (ELP-mmpL-CPP-rho and ELP-CPP-rho) with MMP-2, the reactant was run on SDS-PAGE and analyzed by both silver staining and fluorescence scanning (Figure 2B). Silver-stained gels revealed that MMP-digested ELP-mmpL-CPP-rho produced two bands (lane in the left panel); the upper one for ELP (60 kDa) and the other for cleaved CPP-rho (3 kDa), while ELP-CPP-rho digestion produced only one band (lane in the left panel) which represents undigested ELP-CPP-rho. However, when the gel was scanned for fluorescence, each reactant showed only one band. Since rhodamine was conjugated to the C-terminal of the CPP (Tat peptide), MMP-digestion produced one single fluorescent band (CPP-rho, 3 kDa) without ELP (lane in the right panel), while a band of undigested ELP-CPP-rho fluoresced at around 63kDa, as with the silver-stained gel (lane in the right panel).

MMP-2 digestion increases the cellular uptake of CPP-rhodamine in breast cancer cells

MMP-digestion will produce CPP-rhodamine (rho), which is smaller than the whole construct, ELP-mmpL-CPP-rho. This small size would be a primary contributor for facilitated uptake by cells. Cells treated with MMP-digested ELP-mmpL-CPP-rho and ELP-CPP-rho, respectively, were analyzed for uptake ability via flow cytometry. In Figure 3A, cells treated with cleaved CPP-rho (from ELP-mmpL-CPP-rho) showed up to five times higher uptake rates than the ELP-CPP-rho treated group in three cancer cell lines. This improved cellular uptake was also evident in observation with a fluorescence microscope (Figure 3B).

Cleaved CPP-Dox kills breast cancer cells more efficiently than non-cleaved ELP-CPP

Rhodamine was replaced by doxorubicin to investigate whether improved uptake of cleaved CPP will contribute to cytotoxocity. Figure 3C compares the cytotoxicities of MMP-2 digested ELP-mmpL-CPP-Dox and ELP-CPP-Dox against three cancer cell lines. Improved cytotoxicity was observed in MMP-2 digested ELP-mmpL-CPP-Dox treated cells over ELP-CPP-Dox. These results suggest that MMP digestion of ELP-mmpL-CPP-Dox results in increased uptake of cargo molecules and facilitated death of cancer cells by cleaved CPP-Dox.

Cleaved CPP-Dox deposits in and kills Dox-resistant cancer cells.

To investigate whether cleaved CPP-Dox is able to penetrate and kill even Dox-resistant cancer cells, comparison of cytoxotoxicities and uptake rates of MMP-cleaved CPP-Dox were made between Dox-resistant cells (NCI-ADR, MESSA-5DX) and Dox-sensitive cells (MCF7, MES-SA).

Figure 4A shows the validated Dox resistance in MCF/ADR and MESSA-5DX, and cleaved CPP-Dox from ELP-mmpL-CPP-Dox showed more cell killing than ELP-CPP-Dox at 4uM Dox equivalence. Confocal microscopic images of MCF/ADR cells show that cleaved CPP-Dox from ELP-mmpL-CPP-Dox were taken up by MCF/ADR more than the other groups; free Dox and ELP-CPP-Dox (Figure 4B). This was also confirmed by flow cytometry (Figure 5C). The uptake rate of MMP-digested CPP-Dox in MCF/ADR was almost doubled compared with those of free Dox and ELP-CPP-Dox. These results suggest that MMP-cleaved CPP-Dox can penetrate and kill even Dox-resistant cancer cells probably with help of a CPP (Tat peptide). One limitation in this experiment to mention is that 4uM of doxorubicin equivalent dose is the maximum concentration that can be reached from the current cleavage assay protocol; further optimization of the protocol may enable generation of higher concentration of each drug and calculation of IC50 to compare the cytotoxicity of each treatment.

MMP-releasing HT-1080 can cleave ELP-mmpL-CPP-rho and take up cleaved CPP-rho.

Given that MMP-cleaved CCP-dox can inhibit proliferation in Dox-resistance breast cancer cell lines, this ELP-mmpL-CPP-Dox system was further validated using HT-10180, a fibrosarcoma cancer cell line, producing endogenous MMP2 and MMP9. This experiment shows that the ELP-mmpL-CPP construct can also be digested by endogenous MMP enzyme and release CPP-cargo molecules. MMP-releasing HT1080 cells were incubated with either ELP-mmpL-CPP-rho or ELP-CPP-rho for 4hrs, and each group of treated cells was processed either for flow cytometry or fluorescence microscopy. In flow cytometry, cells

incubated with the ELP-mmpL-CPP-rho group had twice the rhodamine signal than did the ELP-CPP-rho group. However, this increased uptake was reversed by pretreatment with GM6001, an MMP catalytic inhibitor (Figure 5A). This finding was further confirmed by fluorescence microscopy, with the rhodamine particles being found in the nucleus of HT1080 cells treated with ELP-mmpL-CPP-rho (Figure 5B). Uptake of these particles, as in the flow cytometry experiment, were also abolished by GM6001 pretreatment. GM6001 prevents MMP digestion, and undigested ELP-mmpL-CPP-rho was likely washed off the cells during the rinsing step. These results demonstrate that ELP-mmpL-CPP-rho is digested by intrinsic MMP released from HT-1080, and the resultant cleaved CPP-rho penetrates the HT-1080 cells.

Discussion

Our tumor-targeted drug delivery system using ELP delivers anticancer cargo molecules specifically to the tumor site by exploiting the enhanced permeation and retention (EPR) effect along with the active thermal targeting approach (15-17, 21). This thereby increases the relative concentration of the cargo drugs in tumors and improves the therapeutic index of the drugs, alleviating unacceptable toxicity to the patients (12, 22). A striking example of this targeting can be found in previous research (21) in which fluorescently-labeled CPP-ELPs were administered into S2013 tumor-bearing mice. One group of animals received hyperthermic treatment with infrared (IR) lasers on tumors immediately after injection of CPP-ELP so that the temperature in the tumor core reached 42 °C, while the other group were exempt from hyperthermic treatment. This study demonstrated that the IR heating of tumors created 2–3 times greater tumor accumulation of CPP-ELP as well as the penetration of this protein into the tumor tissues. Given the thermo-responsive behavior of ELP, the aggregation of CPP-ELP in hyperthermic tumors resulted in an increase of the construct's concentration in the tumors. These results strongly suggest that ELP preferentially accumulates in tumors in response to local hyperthermia.

We have further renovated the ELP system in this study to release payloads in response to additional external stimuli, matrix metalloproteinases. This novel system is comprised of four components; ELP, MMP-2-cleavable linker, CPP (Tat peptide) and doxorubicin as a payload. The linker is a substrate of MMP-2, designed to be cleaved by MMP-2 so that the ELP system can eventually release a complex of the payload (doxorubicin) and a CPP (CPP-Dox) in tumor tissues. Involvement of MMPs, zinc-containing endopeptidases, in cancer biology has been extensively discussed in a variety of publications (23-26). Especially, increased expression and activity of MMP-2 and MMP-9 subtypes in tumors are known to be related to the degradation of basement membranes, an essential step in tumor invasion, and enhancing angiogenesis. For example, Tutton et al. reported that MMP-2 expression was significantly increased in colorectal cancer tissues compared to matched normal colon as measured by ELISA (27). High levels of MMPs in tumors will facilitate the release of CPP-Dox out of the renovated ELP complex and provide the additional secondary tumor targeting opportunity compared to the previous ELP delivery system. This system, thus, becomes a triple-targeting strategy when used along with the EPR effect and local hyperthermia. Specifically, this cleavable ELP construct still contains the ELP molecule until just before being digested by MMPs at tumor tissues. This late cleavage process will allow CPP-Dox to benefit from ELP by EPR and thermo-targeting. ELP is expected to allow the proposed construct to be initially

targeted to the tumor site by local application of mild heat. Then, ELP-mmpL-CPP-Dox will be fully digested by MMP to release CPP-Dox (Figure 1B), followed by improved cellular uptake by cancer cells and increased cancer cell death.

This MMP-cleavable system displays a couple of other advantages in delivering chemotherapeutic molecules. First, when the MMP-cleavable ELP-CPP-drug is digested by MMP in tumor tissue, small fragments (CPP-Dox) will be produced. Since the molecular weight of the released CPP-Dox (< 3kDa) is twenty times less than the parental ELP construct (60kDa), it will quickly infiltrate into adjacent tumor cells, as can be seen in other studies (28, 29). This hypothesis was examined by cell uptake assays in this study. Cells treated with CPP-rho, which is a digested product from ELP-mmpL-CPP-rho, showed more rhodamine uptake than the cells treated with undigested ELP-CPP-rho. It was also demonstrated in Figure 5 that the ELP-mmpL-CPP-rho can be digested by endogenous MMPs and taken up by HT1080 cells. Reversal of this uptake by GM6001, an MMP inhibitor, indicates that the cell uptake of rhodamine by HT1080 relied on the catalytic activity of MMP. This increased uptake was reflected in the enhanced cytotoxicity of MMP-cleaved CPP-Dox in breast cancer cells. After MMP-2 digestion, ELP-mmpL-CPP-Dox killed more cancer cells than ELP-CPP-Dox (Figure 3C).

A second advantage of this system is that the released CPP-Dox still takes advantage of the abilities of the CPP to facilitate uptake by the cells and to penetrate physiological barriers like the blood-brain barrier. More importantly, there are an increasing number of studies reporting the role of CPPs on overcoming the multidrug resistance (MDR) of cancer cells, which has been one of main hurdles that doxorubicin has faced. The use of doxorubicin, one of the most effective chemotherapy agents since the 1960s, has been compromised by the development of MDR in patients (30, 31). MDR involves increased efflux, decreased uptake, and enzymatic drug metabolism (e.g. glutathione S-transferase) of chemotherapeutic drugs such as doxorubicin (32). An elevated expression of active drug transporters in cancer cells is known to be a major resistance mechanism (33). The coupling of chemotherapeutic drugs to peptides such as CPPs has been suggested as the solution for these problems, since this strategy may alter the cellular uptake pathway and circumvent ABC-transporter-mediated drug efflux, allowing drugs to accumulate at high concentrations in drug-resistant cells, leading to an improved therapeutic index and fewer adverse effects (29, 34-36). Specifically, CPP-Dox developed by Liang et al. (36) showed a 59% uptake rate in Dox-resistant MCF7 cells, while 90% of free dox was lost during cell penetration, leading to considerable improvement in the IC50 of doxorubicin. In line with these reports, our current study also demonstrates that cleaved CPP-Dox showed facilitated cellular uptake by Dox-resistant NCI-ADR in comparison with free Dox and ELP-CPP -Dox (Figure 4C). Like Dox-sensitive breast cancer cells, this increased uptake also led to enhanced cytotoxicity of cleaved CPP-Dox against NCI-ADR and MESSA-5DX (Figure 4B).

Conclusions

In summary, the modified ELP-CPP-Dox is cleaved by incubation with intrinsic or extrinsic MMP enzyme. MMP-digestion produces CPP-Dox (rhodamine), which shows better membrane penetration and induces

more cancer cell death *in vitro*. This CPP-complexed Dox released from ELP kills even dox-resistant cells more efficiently than both free doxorubicin and non-cleaved ELP-CPP-Dox.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

Drazen Raucher is a founder/owner of a 'Thermally Targeted Therapeutics, Inc.'.

Felix Kratz is Vice president of CytRx Pharmaceuticals.

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Authors' contributions

JR conceived and planned the experiments. JR, FK and DR discussed the results and contributed to the final manuscript.

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Abbreviations

ELP: Elastin-like polypeptide

MMP: Matrix metalloproteinase

CPP: Cell penetrating peptide

Dox: Doxorubicin

MDR: Multidrug resistance

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Table

Table 1. Construct Sequences

Construct	Sequence
MMP-cleavable ELP-mmpL-CPP	ELP-(PLGALG)-CPP-(GGC)3
MMP-uncleavable ELP-CPP	ELP-GGGGGG-CPP-(GGC)3

Figures

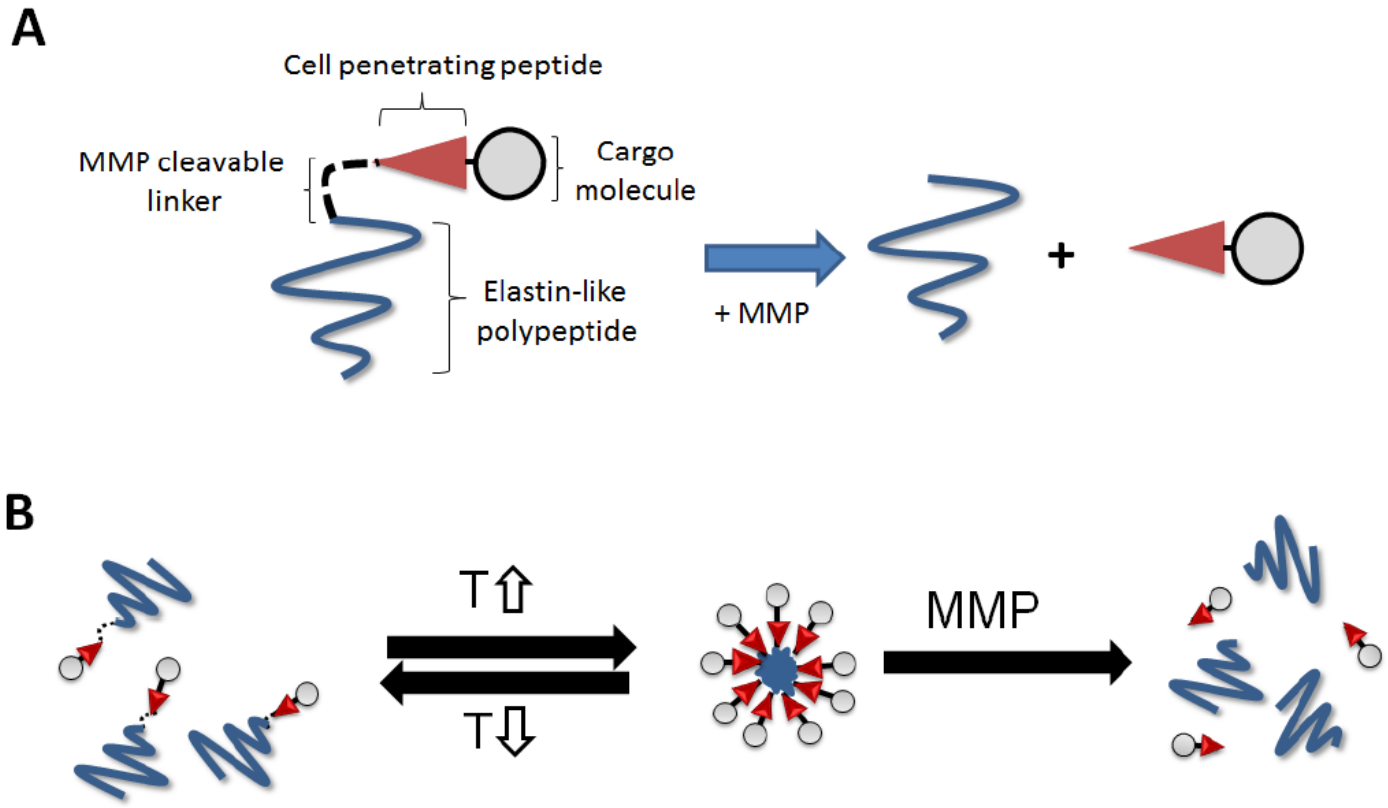

Figure 1

ELP drug delivery system A. A proposed ELP system consists of elastin-like polypeptide, MMP-cleavable linker, cell penetrating peptide and cargo molecules. The constructs are digested by MMP releasing CPP-cargo molecule. B. A hypothetical model proposed in this system. The ELP constructs can form aggregates and release CPP-cargo molecule in hyperthermic tumors.

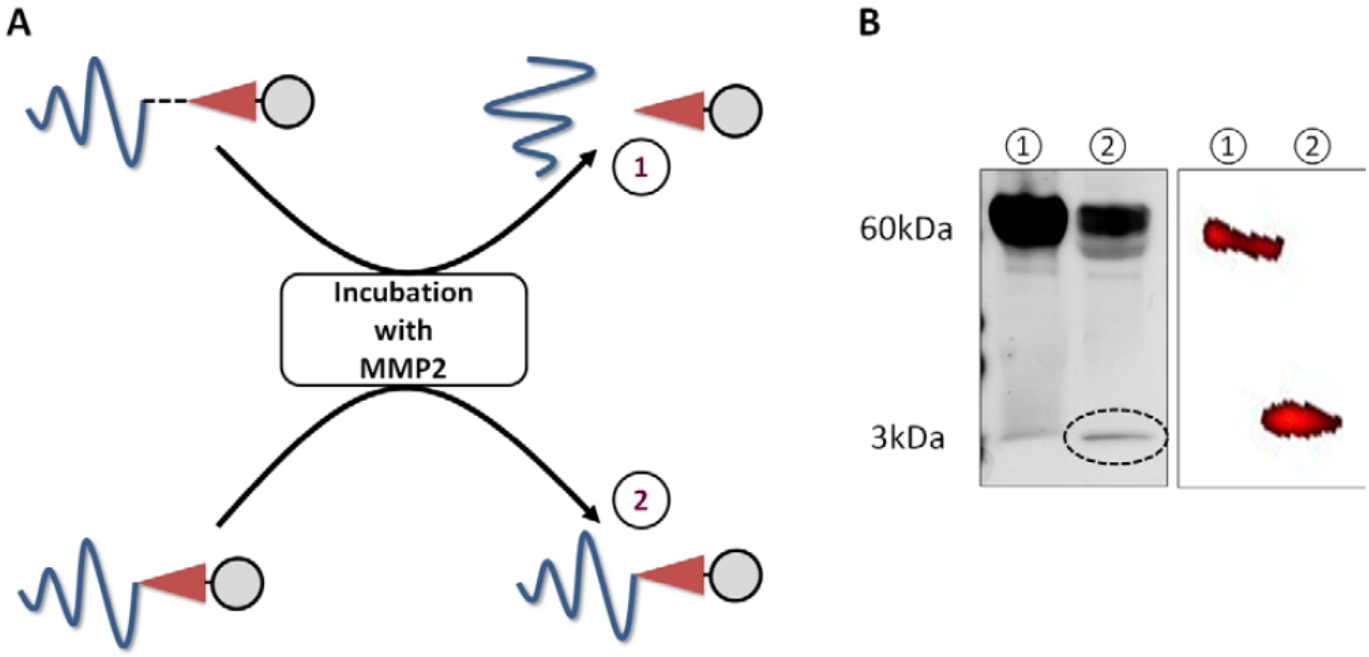

Figure 2

MMP-2 digestion of ELP-mmpL-CPP. A. ELP-mmpL-CPP-rhodamine and ELP-CPP-rhodamine were incubated with MMP-2 for 4hr in ZnCl2 buffer (pH 7). B. MMP-2 incubation of the constructs produced ELP-CPP-Dox (63KDa, upper band) and CPP-rhodamine (3kDa, lower band). Left panel: Silver-stained gel, Right panel: fluorescence-scanned gel.

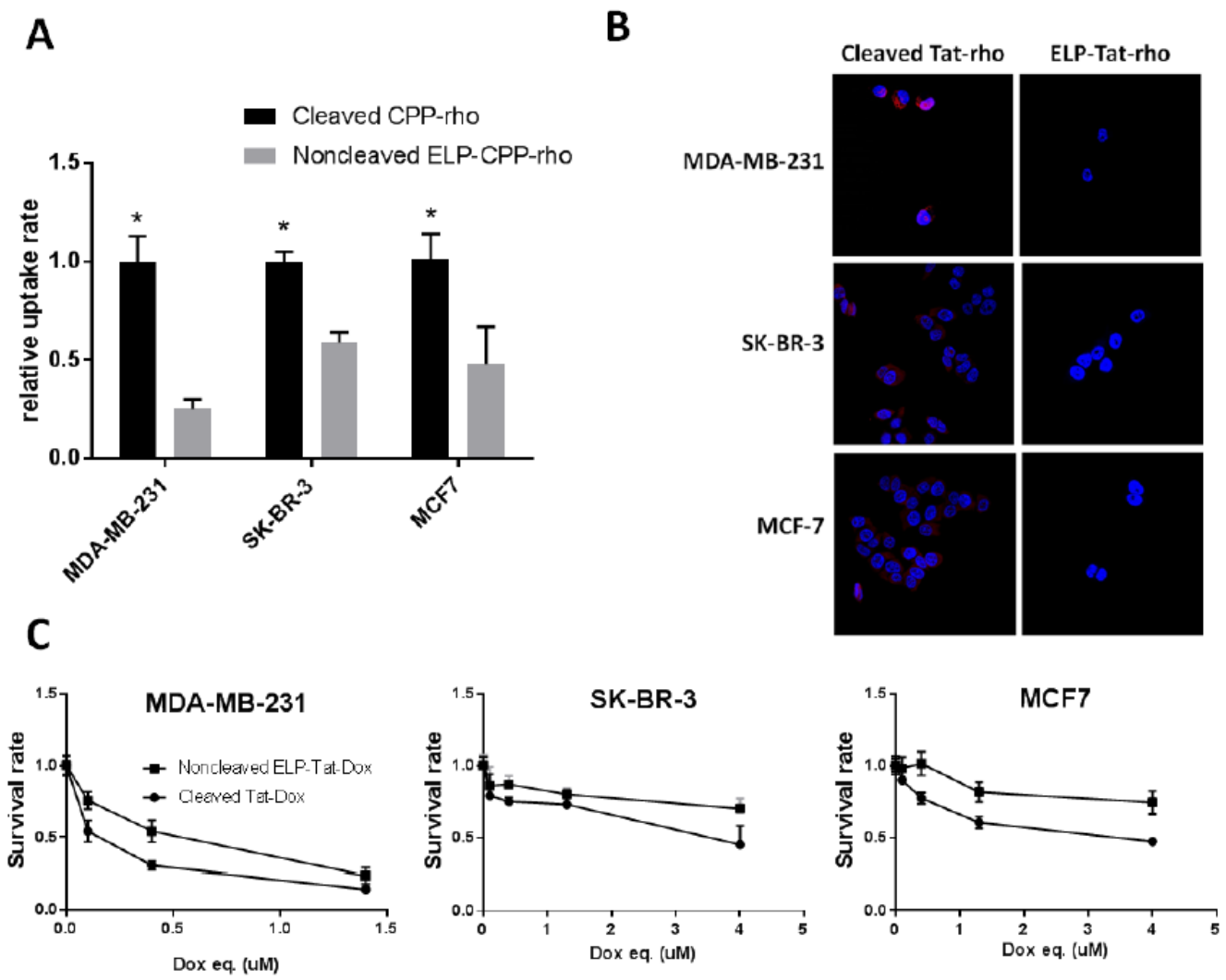

Figure 3

Cellular uptake rate of cleaved CPP-rhodamine in breast cancer cells. A. Each cellular uptake rate was measured through flow cytometry (*; P<0.05). B. Merged image of Dox (red) and DAPI (blue). C. Cytotoxicity of cleaved CPP-Dox on breast cancer cells. Cells were treated with ELP-CPP-Dox and ELP-mmpL-CPP-Dox, both digested by MMP incubation.

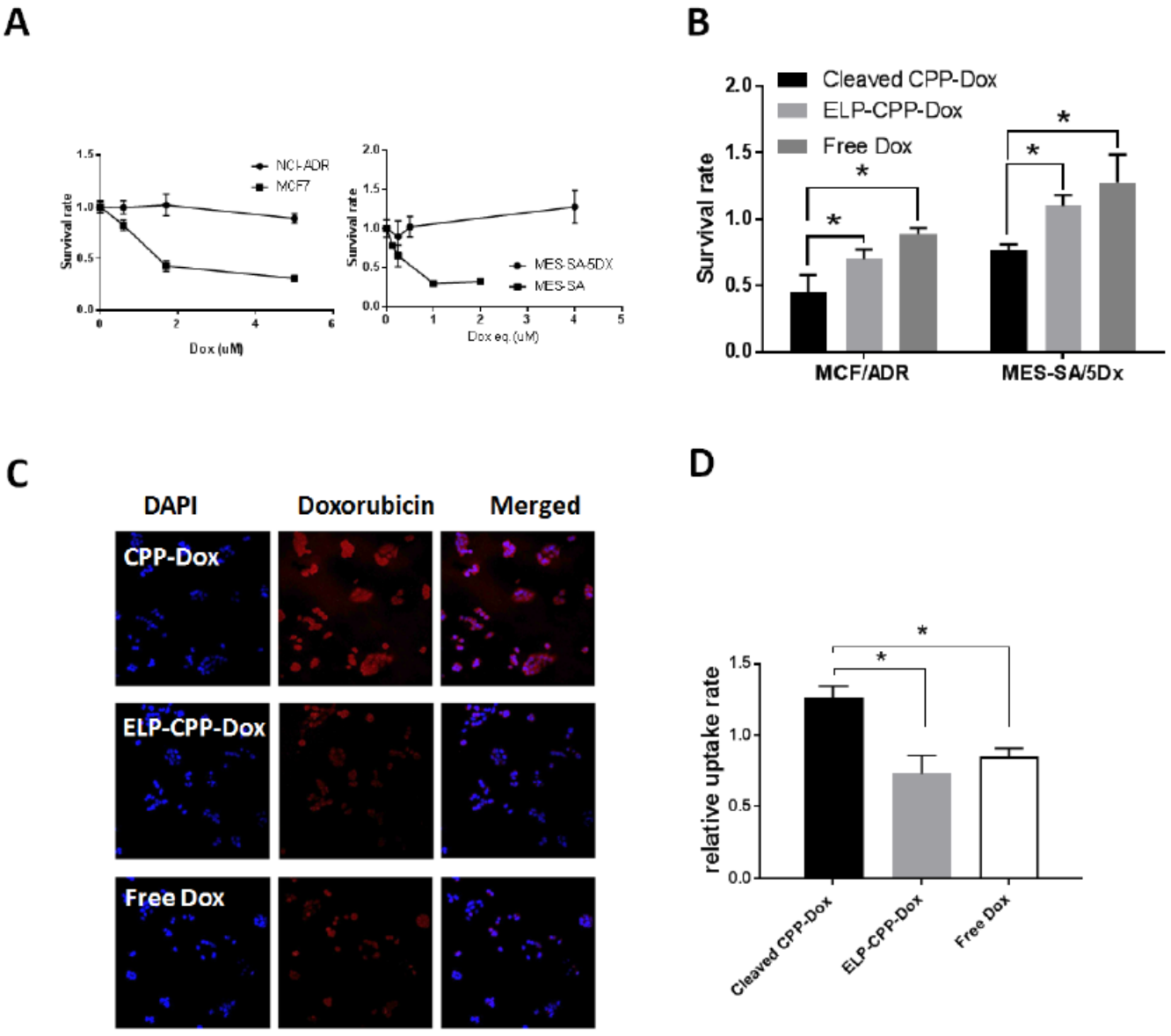

Figure 4

Cytotoxicity of CPP-Dox against Dox-resistant cancer cells. A. Free Dox kills Dox-sensitive cancer cell lines (MCF7 and MES-SA), while it spares Dox-resistant MCF/ADR and MESSA-5DX. B. Cytotoxicities of CPP-Dox and ELP-CPP-Dox in MCF/ADR and MES-SA/5DX at 4uM Dox equivalence. C. Confocal microscopic images show that CPP-Dox penetrated into MCF/ADR. D. Flow cytometry, 60% increased uptake in CPP-Dox in comparison with ELP-CPP-Dox and free Dox. *: P<0.05

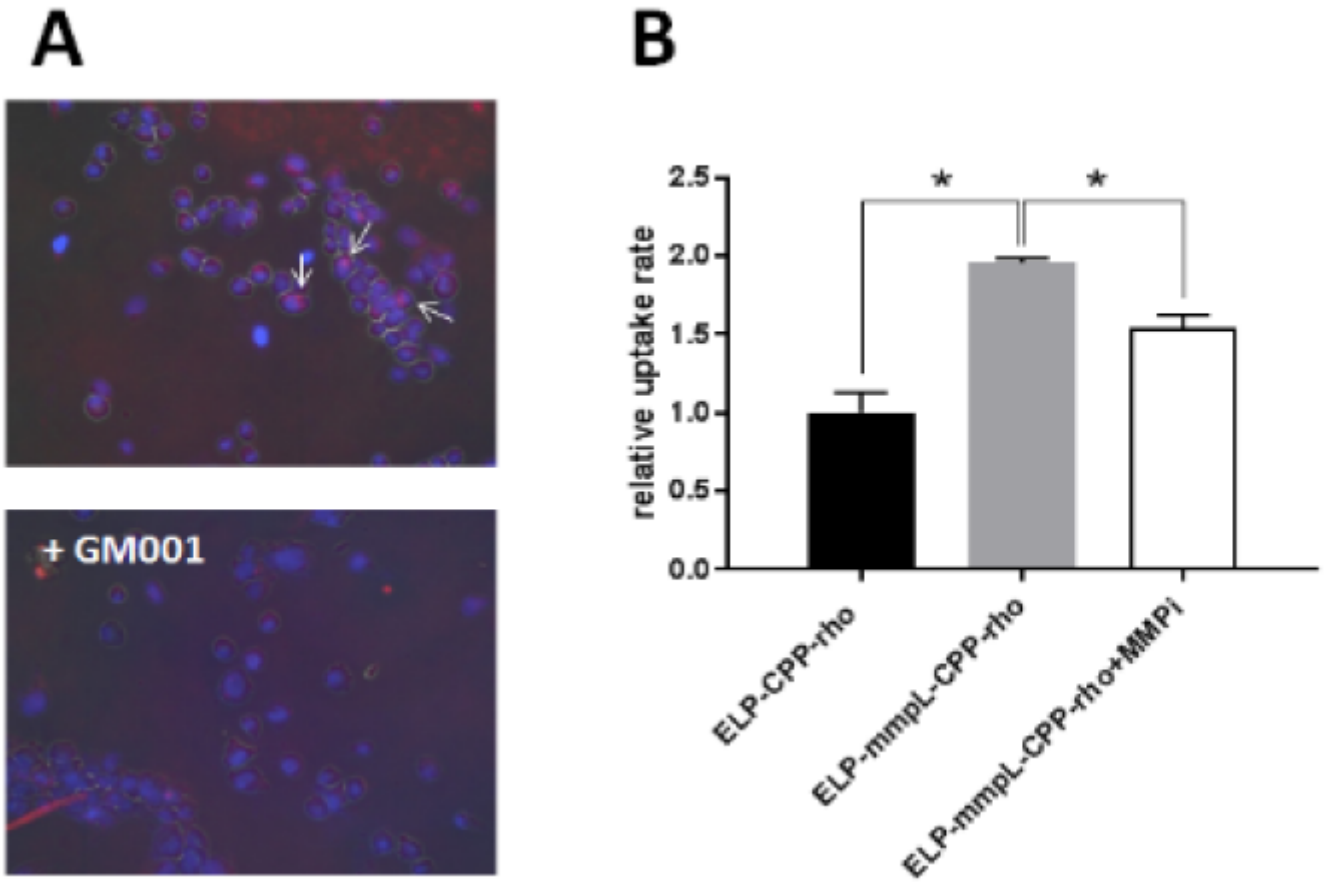

Figure 5

Cellular uptake rate of CPP-rhodamine in MMP-expressing HT-1080. A. Localization of CPP-rho (fluorescence microscopy, 20X) in cultured HT-1080. The arrows indicates the CPP-rhodamine in the cells. B. Flow cytometry showing increased uptake in cleaved CPP-rho in cells.