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A thermally targeted c-Myc inhibitory polypeptide inhibits breast tumor growth

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Abstract

Although surgical resection with adjuvant chemotherapy and/or radiotherapy are used to treat breast tumors, normal tissue tolerance, development of metastases, and inherent tumor resistance to radiation or chemotherapy can hinder a successful outcome. We have developed a thermally responsive polypeptide, based on the sequence of Elastin-like polypeptide (ELP), that inhibits breast cancer cell proliferation by blocking the activity of the oncogenic protein c-Myc. Following systemic administration, the ELP – delivered c-Myc inhibitory peptide was targeted to tumors using focused hyperthermia, and significantly reduced tumor growth in an orthotopic mouse model of breast cancer. This work provides a new modality for targeted delivery of a specific oncogene inhibitory peptide, and this strategy may be expanded for delivery of other therapeutic peptides or small molecule drugs.

Keywords

Elastin-like polypeptide; c-Myc; Thermal targeting; Therapeutic peptide; Breast cancer

1. Introduction

Therapeutic peptides have great potential for cancer treatment due to their ability to target molecules for which no small molecule drugs are available [1,2]. However, the utility of therapeutic peptides is limited by their susceptibility to degradation and poor tumor penetration *in vivo* [3]. In order to make peptides viable as therapeutic agents, a suitable carrier is needed that can stabilize them from degradation in serum and mediate their penetration across biological membranes [1,2].

Our previous work has used Elastin-like polypeptide (ELP), modified with the Bac cell penetrating peptide (CPP) [4,5], to deliver a peptide inhibitor of transcriptional activation by c-Myc to breast cancer cells *in vitro* [5,6]. ELP is a thermally responsive biopolymer that

reversibly forms aggregates at temperatures just above body temperature [7]. ELP can be concentrated at a tumor site by focusing mild hyperthermia at the target region [8,9]. The c-Myc inhibitory peptide is based on the sequence of helix 1 (H1) of the helix-loop-helix domain of c-Myc, and it functions by interfering with the interaction of c-Myc and its dimerization partner Max [10,11]. We have shown that the Bac-ELP-H1 polypeptide can enter tumor cells and localize to both the cytoplasm and the nucleus [5], and its cellular uptake and resulting antiproliferative effect are enhanced by inducing aggregation of the polypeptide with mild hyperthermia applied during treatment [5,6].

Previous studies have shown that ELP levels in tumors can be enhanced using focused hyperthermia [8,9,12]. Using fluorescence videomicroscopy, Meyer et al. demonstrated that hyperthermia could enhance the uptake of ELP in tumors by twofold, and a portion of the enhancement was attributable to the thermally induced aggregation of ELP [12]. In a follow up study using radiolabeled ELP, Liu et al. demonstrated a similar 1.8-fold enhancement of ELP deposition in flank tumor xenografts, and of the 1.8-fold enhancement, 1.5-fold was attributed to thermally induced ELP aggregation (with the rest attributed to effects of hyperthermia on vascular permeability) [8]. Also, cycling the tumor between periods of hyperthermia and periods of normothermia has been found to be superior to a longer, constant hyperthermia treatment for ELP targeting [9]. However, no evaluation of CPP-fused ELPs has been carried out *in vivo*. And, more importantly, no demonstration of the ability of ELP to deliver a therapeutic agent and inhibit tumor progression in a thermally targeted manner has been published to date.

In this study, we used an orthotopic model of breast cancer generated by implantation of E0771 medullary breast adenocarcinoma cells [13,14] into C57BL/6 mice to evaluate the ability of Bac-ELP-H1 to be thermally targeted to tumors and to reduce tumor growth *in vivo*. This model was chosen because the cells are syngeneic with C57BL/6 mice, allowing us to monitor tumor progression and treatment effects in animals with a functioning immune system. This is important when evaluating protein and peptide-based therapeutics due to their potential for immunogenicity. The E0771 tumor model was also chosen because it is aggressive, metastatic, and closely mimics the human disease [15]. This article reports the pharmacokinetics, tumor and organ biodistribution, effective tumor thermal targeting, and significant inhibition of tumor progression by the Bac-ELP-H1 polypeptide.

2. Materials and methods

2.1. Purification and fluorescent labeling of polypeptides

The Bac-ELP-H1 polypeptide has the amino acid sequence MRRIRPRPPRLPRPRPLPFPRPGGCYPG-(VPGXG)*n*-WPGSGNELKRAFAALRDQI. Bac-ELP1-H1 contains V, G, or A at the X position in a 5:3:2 ratio, respectively, and *n* is 150. Bac-ELP2-H1 contains V, G, or A at the X position in a 1:7:8 ratio, respectively, and *n* is 160. ELP polypeptides were expressed in *E. coli* and purified by thermal cycling as described in [6], and labeled on a unique cysteine residue with tetramethylrhodamine-5-maleimide or AlexaFluor® 750 C₅-maleimide (Invitrogen) [6]. Stability of the attached label was determined by incubating the labeled Bac-ELP1-H1-Rho polypeptide in fresh mouse plasma for various times at 37 °C. After incubation, the Bac-ELP1-H1 polypeptide was thermally precipitated from the mixture, and the absorbance of any unconjugated rhodamine label remaining in the plasma was measured at 543 nm using a NanoDrop spectrophotometer. Absorbance values were compared to 0 h incubation as baseline and reported as % of the un-precipitated protein sample.

2.2. Tumor implantation

E0771 medullary breast adenocarcinoma cells were obtained from Dr. F.M. Sirotnak at Memorial Sloan-Kettering Cancer Center (New York) in 2008. Cells were maintained in RPMI-1640 medium (Mediatech) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 100 U/mL penicillin, 100 µg/mL streptomycin, and 25 µg/mL amphotericin B (Invitrogen). E0771 tumors were established by injection of 1×10^6 cells suspended in 200 µL PBS into the mammary fat pad of female C57BL/6 mice (NCI). All animal manipulations were performed in accordance with the National Institutes for Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Mississippi Medical Center's Institutional Animal Care and Use Committee.

2.3. Tumor hyperthermia

Tumor heating was performed by illumination of the tumor using the SLD/LED cluster of a Laser Sys-Stim 540® (Mettler Electronics). The tumor was heated by continuous illumination for 20 min, followed by a 10 min cooling period, and this protocol was repeated for 4 cycles. The area surrounding the tumor was shielded from illumination, and the tumor core temperature was measuring using a 30 ga needle thermocouple connected to a HHM290 Supermeter (Omega Engineering). Skin temperature over the tumor was monitored using an infrared thermometer, and body temperature was monitored with a rectal probe and maintained at 37 °C using a homeothermic blanket (Harvard Apparatus).

2.4. Pharmacokinetics

Mice bearing 250 mm³ E0771 tumors were anesthetized with isoflurane, and a cannula was placed in the femoral artery. Rhodamine-labeled Bac-ELP1-H1 or Bac-ELP2-H1 (100 mg/kg) was administered by IP injection or IV injection into the femoral vein. Tumors in the heated group were heated by thermal cycling beginning immediately after IV injection or 1 h after IP injection using the thermal cycling protocol described above. 30 µL of blood was sampled at the indicated time points over a period of 3 h using the arterial catheter. Blood was collected in heparinized hematocrit tubes and centrifuged ($13,000 \times g$, 5 min) to separate the plasma from the cells. 1 min prior to euthanasia, 500 kDa FITC-dextran (25 mg/kg, Sigma) was injected IV to mark the perfused vasculature. Polypeptide fluorescence in the plasma was determined using a fluorescence plate reader (Bio Tek), and a standard curve was generated using known quantities of the injected polypeptide. Fluorescence data were fit to the standard curve to calculate plasma levels (µg/mL) at each sampling point. Plasma clearance data were fit to a two-compartment pharmacokinetic model using Microcal Origin. Plasma concentrations with time were fit to the relationship:

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$

where:

$$A = \frac{x_0(\alpha - k_{ip})}{V_c(\alpha - \beta)}$$

And:

$$B = \frac{x_0(k_{ip} - \beta)}{V_c(\alpha - \beta)}$$

From these data and the dose (D), the following parameters were calculated according to the relationships shown:

$$C_0 = A + B$$

$$V_c = \frac{D}{C_0}$$

$$Cl = k_{el} V_c$$

$$AUC = \frac{D}{Cl}$$

$$k_{tp} = \frac{A\beta + B\alpha}{A + B}$$

$$k_{pt} = \alpha + \beta - k_{tp} - k_{el}$$

$$k_{el} = \frac{\alpha\beta}{k_{tp}}$$

$$t_{\frac{1}{2}, dist} = \frac{\ln[2] \left[k_{el} + k_{pt} + k_{tp} - \left((k_{el} + k_{pt} + k_{tp})^2 - 4k_{el}k_{tp} \right)^{0.5} \right]}{2k_{el}k_{tp}}$$

$$t_{\frac{1}{2}, term} = \frac{2 \ln[2]}{k_{el} + k_{pt} + k_{tp} - \left((k_{el} + k_{pt} + k_{tp})^2 - 4k_{el}k_{tp} \right)^{0.5}}$$

2.5. Quantitative fluorescence analysis

Acute biodistribution studies were performed using tumors and organs harvested 3 h after the IP or IV administration described above. Tumors and major organs were harvested at necropsy, rapidly frozen, cut into 15 μ m sections using a cryo-microtome, and mounted onto slides. Fluorescence standards were made by freezing known quantities of the injected protein and cutting discs to the same thickness as tissue sections. Tissue sections and standards were scanned using a ScanArray Express slide scanner (Perkin Elmer). Fluorescence intensity of sections and standards was determined using Image J software,

and % of injected dose/gram (%ID/g) was calculated by fitting the tumor or organ fluorescence intensity to the standard curve after subtraction of autofluorescence from control, saline injected organs. Tumor and organ levels were averaged for all animals ($n = 6$ mice/group), and data represent the mean $\pm$ s.e.

2.6. In vivo fluorescence imaging

Tumor polypeptide levels in live animals over time were determined after IV administration of AlexaFluor750-labeled Bac-ELP1-H1 (100 mg/kg) by fluorescence whole-animal imaging with an IVIS Spectrum (Caliper). Polypeptide, or saline control, were injected IV via the femoral vein in animals with or without E0771 tumors, and, in heated groups, the tumor was heated using the thermal cycling protocol described above. Images were collected at the indicated time points, and tumor fluorescence was determined using Living Image software (Caliper). Fluorescence intensity of non-tumor bearing mammary fat pads was subtracted from the tumor fluorescence, the corrected tumor fluorescence was averaged for all animals ($n = 5$ mice / group), and the data represent the mean $\pm$ s.e.

2.7. Tumor reduction

Tumor reduction studies were performed by giving 7 daily IP injections of 200 mg/kg of the indicated polypeptide, or saline control, with or without hyperthermia. Treatment was initiated when tumors reached 150 mm³ (Day 0), and tumor volume and body weight were monitored daily. Tumor volume was calculated using the formula:

$$V = \frac{w^2 l}{2}$$

Tumor weight was determined at necropsy.

2.8. Statistical analysis

Acute biodistribution data and tumor reduction data were analyzed using a one-way ANOVA and post hoc Bonferroni multiple comparisons. The difference between heated and unheated tumor fluorescence in the IVIS experiment were compared using a student's t-test. Statistics were calculated using Analyze-It for Microsoft Excel.

3. Results

3.1. Externally applied tumor hyperthermia

To apply hyperthermia to rodent tumors, we illuminated them with infrared light (IR, 950 nm) generated from the SLD/LED cluster of a Laser Sys-Stim 540 (Mettler Electronics, Anaheim, CA). This device allows penetration of IR light into the tumor tissue, and the light source can be applied directly over the tumor site while shielding surrounding areas from illumination, providing a focused source of hyperthermia. A previous study demonstrated that ELP deposition in tumor is enhanced by cycling between periods of mild hyperthermia and cooling periods [9]. Mild hyperthermia (40–42 °C) causes ELP to aggregate and accumulate in the tumor vasculature, and upon return to normothermia, ELP re-dissolves and diffuses down the concentration gradient that has formed from the vasculature into the extravascular space [9]. As shown in Fig. 1, the core temperature in the tumor could be raised from 33 °C to 41 °C within 20 min by applying IR light. When the light source was removed, the tumor core temperature returned to body temperature very quickly. Thermal cycling between the desired hyperthermia temperature of 40–42 °C and body temperature was achieved repeatedly with 20 min cycles of IR illumination, followed by 10 min cooling periods. During this process, the mouse's body temperature did not rise above 37 °C, and the

skin over the tumor site was not significantly heated by the IR light, presumably because absorbance of the light over some depth is required for the temperature to increase. This schedule of 4 20 min heating cycles followed by 10 min cooling cycles was used for hyperthermia application throughout this study.

3.2. Bac-ELP-H1 plasma stability and pharmacokinetics

Before beginning *in vivo* evaluation of Bac-ELP-H1-Rho pharmacokinetics and biodistribution, we insured that the fluorescent label was stable in plasma. The labeled Bac-ELP1-H1-Rho polypeptide was incubated in fresh mouse plasma for various time periods at 37 °C, then the polypeptide and attached label were precipitated using two rounds of thermal aggregation and centrifugation. The unique absorbance of the rhodamine label at 543 nm was examined in the remaining plasma to determine whether any dye had been released from the polypeptide. As shown in Fig. 2A, the covalently attached rhodamine label was quite stable in plasma, as only 9% was released over the 3 h time course required for our experiments. Even over 24 h, only 20% was released.

Plasma levels were examined after administration of rhodamine-labeled Bac-ELP-H1 by quantitative fluorescence analysis. Both the intravenous (IV) and intraperitoneal (IP) routes were examined. Following IV injection, the thermally responsive Bac-ELP1-H1-Rho polypeptide was cleared from circulation (Fig. 2B), and the data were well described by a two-compartment pharmacokinetic model (Materials and Methods). The terminal half-life was found to be 102.0 ± 9.2 min and the plasma AUC was 9713.5 ± 1393.9 $\mu\text{g min/mL}$ (Table 1). When the tumor was heated for 2 h after injection using the heat cycling protocol described above, the distribution half-life was slightly faster, but the difference was not statistically significant. The terminal half-life was unaffected by tumor hyperthermia. (Table 1). Following IP administration, the polypeptide slowly entered systemic circulation. 90 min after injection, the plasma level of Bac-ELP-H1-Rho was equal to that observed after IV injection, and the plasma levels peaked at 150 min post injection (Fig. 2B). These data demonstrate that the IP route leads to equivalent plasma levels as obtained by IV injection within approximately one half-life, and the IP route may be a viable method for administering multiple chronic doses.

3.3. Tumor deposition and biodistribution of Bac-ELP-H1

Tumor and organ biodistribution was determined 3 h after IV or IP administration of rhodamine-labeled Bac-ELP-H1 by quantitative fluorescence analysis of cryo-sections using a slide scanner. As shown in Fig. 2C, Bac-ELP1-H1-Rho accumulated to detectable levels in tumor following either IV or IP administration, and tumor levels of the polypeptide were visibly higher in hyperthermia treated tumors. Tumor levels of the thermally responsive Bac-ELP1-H1-Rho polypeptide trended higher by 1.57-fold and 1.52-fold with hyperthermia treatment following injection by the IV and IP routes, respectively (Fig. 2D). Both administration routes resulted in similar tumor levels. A non-thermally responsive control polypeptide, Bac-ELP2-H1-Rho, was used to determine whether the enhanced tumor deposition was due to polypeptide aggregation or to non-specific effects of hyperthermia. Bac-ELP2-H1-Rho has a similar composition and molecular weight to the active Bac-ELP1-H1-Rho polypeptide, but it does not aggregate at the mild hyperthermia temperatures used in this study [5]. Hyperthermia treatment caused a slight enhancement in tumor levels of Bac-ELP2-H1-Rho relative to unheated tumors, but did not cause an increase in the tumor levels of Bac-ELP2-H1-Rho relative to the Bac-ELP1-H1-Rho treated tumors (Fig. 2E), indicating that the thermal targeting observed with Bac-ELP1-H1 is due mainly to the polypeptide's hyperthermia-induced aggregation. Enhanced polypeptide levels in heated tumors is promising, but in order for thermal targeting to be effective, the polypeptide must escape the vasculature, traverse the extracellular matrix of the tumor, and enter the tumor cells. We

used an IV infusion of 500 kDa FITC-dextran 1 min prior to euthanasia to mark the perfused vasculature of the tumor. Because of its high molecular weight and the short interval between administration and sacrifice, this dextran does not extravasate, and thus serves as a marker for perfused vessels at the time of euthanasia. Fig. 2C shows that much of the Bac-ELP1-H1-rho is present in the blood vessels, but there is a portion of the polypeptide that is not confined to the vascular space. Higher magnification images, using Hoechst 33342 to mark cell nuclei, demonstrate that Bac-ELP1-H1-rho is present in the tumor tissue as well as in the blood vessels (Fig. 2F). FITC dextran intensities were not significantly varied between heated and unheated tumors.

Biodistribution of Bac-ELP1-H1-Rho in the major organs was also determined after IV or IP injection by quantitative fluorescence analysis (Fig. 3). The polypeptide accumulated to high levels in the liver and kidney, and much lower levels were detected in the heart, spleen, lungs, and brain. IP administration led to much lower accumulation in the liver relative to the IV route ($p = 0.0006$, one-way ANOVA, post hoc Bonferroni), spleen levels were higher following IP injection compared to IV injection ($p = 0.0005$), and brain levels were undetectable over background fluorescence following IV injection. Tumor hyperthermia did not significantly affect polypeptide levels in any organ except the spleen.

3.4. In vivo imaging of tumor uptake of Bac-ELP-H1

We used *in vivo* fluorescence imaging to follow polypeptide levels in tumors longitudinally after IV injection. Bac-ELP1-H1, labeled with Alexa750, could be detected in the tumors for up to 48 h after administration (data not shown). As shown in Fig. 4A, Bac-ELP1-H1-Alexa750 accumulated to the highest levels in the tumor, liver, and bladder. Quantitation of the tumor levels, corrected for background fluorescence from non-tumor mammary tissue, indicated that Bac-ELP1-H1-Alexa750 deposition peaked in tumors 6 h after injection, and thermal targeting resulted in as much as 2.8-fold more Bac-ELP1-H1-Alexa750 in the heated tumor than in the unheated tumor (Fig. 4B, $p = 0.007$, student's t-test).

3.5. Inhibition of tumor growth by Bac-ELP-H1

Having demonstrated that thermal targeting results in significantly enhanced levels of Bac-ELP1-H1 in tumors, we tested the ability of the polypeptide, with and without hyperthermia, to reduce tumor growth. Polypeptide injections were initiated when the E0771 tumors reached 150 mm³, and, based on the pharmacokinetic and biodistribution results, polypeptides were given daily at a dose of 200 mg/kg/day via IP administration. As shown in Fig. 5A, the control E0771 tumors reached an average volume of 2500 mm³ within 14 days of the start of treatment. Hyperthermia alone, or the control polypeptide Bac-ELP1 (which lacks the c-Myc inhibitory peptide), had no effect on tumor volume. Bac-ELP1-H1, when given daily for 7 days without hyperthermia, caused a slight reduction in the tumor growth rate, but the results were not statistically significant. However, when Bac-ELP1-H1 treatment was combined with hyperthermia, the tumor volumes were reduced by nearly 70% ($p = 0.0017$, one-way ANOVA, post hoc Bonferroni). All polypeptides caused some body weight loss in the mice during the treatment period (Fig. 5B and D), but the body weight quickly recovered after removal of the treatment, and the mice showed no other signs of toxicity. The non-thermally responsive control polypeptide Bac-ELP2-H1 reduced tumor growth in a similar manner to that observed from Bac-ELP1-H1 treatment without hyperthermia, and addition of hyperthermia had no effect on the antiproliferative effect of Bac-ELP2-H1 (Fig. 5C). These data are consistent with the tumor uptake data, and indicate that the enhanced anti-tumor effect seen with Bac-ELP1-H1 + hyperthermia is due to thermal targeting of the polypeptide. The tumor volume results were confirmed by measuring the tumor mass at necropsy on day 14 after treatment (Fig. 5E).

4. Discussion

Thermal targeting is a novel method for actively targeting therapeutics to tumors that has several advantages. First, hyperthermia increases the vascular permeability and blood flow to tumors [16,17], which can lead to increased drug deposition and extravasation. Second, the hyperthermia-induced aggregation of ELP leads to local accumulation of the polypeptide along with any cargo molecule that is attached [8,9,12]. The technology for application of focused hyperthermia exists or is in development in the clinical setting using high intensity focused ultrasound (HIFU) [18–20], microwave [21–23], or radiofrequency irradiation [24–27]. In this study, we utilized an infrared LED device to induce tumor hyperthermia. This method relies on the absorbance of the IR light by the tumor tissue, and results in hyperthermia temperatures of up to 42 °C in the tumor core. This is a powerful method for hyperthermia application in rodent models with superficial tumors because it is non-invasive and high-throughput. Also, in contrast to previous studies which used water bath immersion to apply tumor hyperthermia in rodent models [8,9,12], the LED illumination method should allow a more specific targeting of the hyperthermia application to the tumor site.

We examined the pharmacokinetics, tumor uptake, and biodistribution of the Bac-ELP-H1-Rho polypeptide after both IV and IP administration. After IV administration, the polypeptide was found to have a plasma half life of approximately 100 min, which is sufficiently long to allow application of hyperthermia to the tumor while the polypeptide is still circulating. The 100 min half life observed for Bac-ELP-H1-Rho is significantly shorter than the 8.37 h half life previously reported for the unmodified ELP polypeptide [8]. This difference is likely due to the addition of the functional CPP and H1 peptides, which give the polypeptide a net positive charge and significantly affect the biodistribution. Also, the different plasma pharmacokinetics may be influenced by the labels used, as it is possible that the covalent conjugation of the rhodamine dye affects the pharmacokinetics relative to the radiolabeled polypeptide used by Liu. In addition to IV administration, the IP route was examined as an alternative to facilitate the administration of multiple chronic polypeptide injections. The plasma levels 100 min after administration were equal to the levels achieved by IV injection, indicating that the IP route may be a suitable alternative. Tumor levels 3 h after injection were similar for both IV and IP administration routes, again indicating that either route is suitable for therapy in this model. Hyperthermia lead to enhancement of tumor levels by about 1.5-fold relative to non-heat treated tumors at the 3 h time point, which is consistent with previous reports of ELP thermal targeting efficacy [8,9,12]. Importantly, when the tumors were examined microscopically, the polypeptide could be detected within the tumor tissue, demonstrating the effectiveness of the CPP for mediating extravasation and cellular uptake.

Though the 1.5-fold enhancement of tumor levels with hyperthermia was consistent with previous literature reports, we were concerned that the timing of the experiment (3 h after injection) was not detecting tumor levels at their peak. Therefore, we followed tumor levels in live animals longitudinally after injection using *in vivo* fluorescence imaging. Indeed, we found that tumor levels peak 6 h after injection, and hyperthermia can enhance Bac-ELP1-H1 tumor levels as much as 3-fold. This experiment also showed that the polypeptide was mostly cleared from tumors within 24 h of injection, which lead us to carry out a daily polypeptide administration schedule for the tumor reduction studies.

Finally, we demonstrated that the combination of Bac-ELP1-H1 injections and hyperthermia treatment was an effective means to slow the progression of breast tumors. Only the full length polypeptide containing the H1 c-Myc inhibitory peptide was tumor inhibitory, suggesting that tumor inhibition is a function of the H1 peptide. We previously assessed the ability of the ELP-delivered H1 peptide to target the c-Myc pathway specifically. We

demonstrated using confocal immunofluorescence microscopy that treatment with the ELP-delivered H1 peptide interrupted the interaction between c-Myc and its dimerization partner Max in breast cancer cells *in vitro*. As a result, the expression levels of several c-Myc/Max responsive genes, but not control genes, were inhibited [6]. The Bac-ELP1 control polypeptide (lacking the H1 peptide) had no effect on tumor progression. In a previous study, we found that Bac-ELP1 did have some toxicity to breast cancer cells *in vitro* [5]. This is true of most CPP-ELPs, and in another previous paper, we have examined this toxicity in detail [28]. The amount of toxicity is both CPP and cell line dependent, most obvious when treatment is combined with hyperthermia, and likely results from effects of the polypeptide on the plasma membrane integrity. Treatment with another CPP-ELP using the Tat CPP caused lactate dehydrogenase leakage from cells and allowed cellular uptake of both propidium iodide and low molecular weight dextrans [28]. In our *in vivo* applications in this study, Bac-ELP showed no inhibition of tumor progression. It may be that the toxic effects of CPP-ELPs are specific to or more potent in the cell culture setting, or, more likely given that the toxicity of Bac-ELP *in vitro* is somewhat mild, that Bac-ELP is not toxic at the dose used for our *in vivo* experiments. In the absence of hyperthermia, the fully functional polypeptide Bac-ELP1-H1 had little effect on tumor progression at the dose used, while the same polypeptide quite significantly inhibited tumor progression when treatment was combined with focused hyperthermia. Bac-ELP2-H1 had a small inhibitory effect on tumor progression (similar to Bac-ELP1-H1 without hyperthermia) under both the hyperthermia and normothermia treatment conditions. These results confirm the findings of the biodistribution study that the tumor deposition and resulting proliferation inhibition is a result of the thermally-induced aggregation of the Bac-ELP1-H1 polypeptide. We believe this to be the first demonstration that thermally targeting the ELP carrier to tumors can result in accumulation of therapeutic cargo at the tumor site, entry of the polypeptide into tumor cells, and inhibition of progression of the thermally targeted tumors.

In summary, this work demonstrates that an ELP-delivered therapeutic peptide can be targeted to the site of a solid tumor using focused hyperthermia, and the polypeptide construct can escape the vasculature, enter the tumor cells, and inhibit their proliferation. This approach is advantageous because (1) the polypeptide is a macromolecule, and it will passively accumulate in tumor tissue due to the enhanced permeability and retention effect [29], (2) the thermally responsive nature makes active targeting of the polypeptide and its payload possible using focused hyperthermia, (3) the peptide inhibitor is specific to c-Myc, making this agent specific to tumor cells overexpressing the target protein, and (4) the polypeptide carrier is genetically encoded, which facilitates the addition of functional peptides and simplifies the purification process. These results demonstrate that ELP is an inert, biodegradable polymer capable of delivering a therapeutic peptide in a targeted, non-toxic manner. This may be applied for breast cancer therapy to treat the tumor bed after resection of a primary mass in order to prevent recurrence from remaining malignant cells. Also, we have previously shown that the ELP-delivered c-Myc inhibitory peptide enhances the potency of topoisomerase II inhibitors doxorubicin and etoposide in a variety of cell lines. This technology could be applied in combination with other drugs or even other targeted peptides to treat inoperable breast recurrences. More broadly, the use of ELP as a vector has great potential to convert promising peptide agonists/antagonists into viable pharmaceutical agents for therapy of any type of solid tumor.

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References

1. Bidwell GL 3rd, Raucher D. Therapeutic peptides for cancer therapy. Part I – Peptide inhibitors of signal transduction cascades. *Expert Opin. Drug Deliv.* 2009; 6:1033–1047. [PubMed: 19637980]
2. Raucher D, Moktan S, Massodi I, Bidwell GL 3rd. Therapeutic peptides for cancer therapy. Part II – Cell cycle inhibitory peptides and apoptosis-inducing peptides. *Expert Opin. Drug Deliv.* 2009; 6:1049–1064. [PubMed: 19743895]
3. Talmadge JE. Pharmacodynamic aspects of peptide administration biological response modifiers. *Adv. Drug Deliv. Rev.* 1998; 33:241–252. [PubMed: 10837664]
4. Sadler K, Eom KD, Yang JL, Dimitrova Y, Tam JP. Translocating proline-rich peptides from the antimicrobial peptide bactenecin 7. *Biochemistry.* 2002; 41:14150–14157. [PubMed: 12450378]
5. Bidwell GL 3rd, Davis AN, Raucher D. Targeting a c-Myc inhibitory polypeptide to specific intracellular compartments using cell penetrating peptides. *J. Contr. Release.* 2009; 135:2–10.
6. Bidwell GL 3rd, Raucher D. Application of thermally responsive polypeptides directed against c-Myc transcriptional function for cancer therapy. *Mol. Cancer Ther.* 2005; 4:1076–1085. [PubMed: 16020665]
7. Urry DW, Luan C-H, Parker TM, Gowda DC, Prasad KU, Reid MC, Safavy A. Temperature of polypeptide inverse temperature transition depends on mean residue hydrophobicity. *J. Am. Chem. Soc.* 1991; 113:4346–4348.
8. Liu W, Dreher MR, Furgeson DY, Peixoto KV, Yuan H, Zalutsky MR, Chilkoti A. Tumor accumulation, degradation and pharmacokinetics of elastin-like polypeptides in nude mice. *J. Contr. Release.* 2006; 116:170–178.
9. Dreher MR, Liu W, Michelich CR, Dewhirst MW, Chilkoti A. Thermal cycling enhances the accumulation of a temperature-sensitive biopolymer in solid tumors. *Cancer Res.* 2007; 67:4418–4424. [PubMed: 17483356]
10. Draeger LJ, Mullen GP. Interaction of the bHLH-zip domain of c-Myc with H1-type peptides. Characterization of helicity in the H1 peptides by NMR. *J. Biol. Chem.* 1994; 269:1785–1793. [PubMed: 8294427]
11. Giorello L, Clerico L, Pescarolo MP, Vikhanskaya F, Salmona M, Colella G, Bruno S, Mancuso T, Bagnasco L, Russo P, Parodi S. Inhibition of cancer cell growth and c-Myc transcriptional activity by a c-Myc helix 1-type peptide fused to an internalization sequence. *Cancer Res.* 1998; 58:3654–3659. [PubMed: 9721875]
12. Meyer DE, Kong GA, Dewhirst MW, Zalutsky MR, Chilkoti A. Targeting a genetically engineered elastin-like polypeptide to solid tumors by local hyperthermia. *Cancer Res.* 2001; 61:1548–1554. [PubMed: 11245464]
13. Dunham LJ, Stewart HL. A survey of transplantable and transmissible animal tumors. *J. Natl. Cancer Inst.* 1953; 13:1299–1377. [PubMed: 13035452]
14. Sugiura K, Stock CC. Studies in a tumor spectrum. I. Comparison of the action of methylbis (2-chloroethyl)amine and 3-bis(2-chloroethyl)aminomethyl-4-methoxymethyl -5-hydroxy-6-methylpyridine on the growth of a variety of mouse and rat tumors. *Cancer.* 1952; 5:382–402. [PubMed: 14905426]
15. Ewens A, Mihich E, Ehrke MJ. Distant metastasis from subcutaneously grown E0771 medullary breast adenocarcinoma. *Anticancer Res.* 2005; 25:3905–3915. [PubMed: 16312045]
16. Lefor AT, Makohon S, Ackerman NB. The effects of hyperthermia on vascular permeability in experimental liver metastasis. *J. Surg. Oncol.* 1985; 28:297–300. [PubMed: 3982038]
17. Fujiwara K, Watanabe T. Effects of hyperthermia, radiotherapy and thermoradiotherapy on tumor microvascular permeability. *Acta Pathol. Jpn.* 1990; 40:79–84. [PubMed: 2160185]

18. Ram Z, Cohen ZR, Harnof S, Tal S, Faibel M, Nass D, Maier SE, Hadani M, Mardor Y. Magnetic resonance imaging-guided, high-intensity focused ultrasound for brain tumor therapy. *Neurosurgery*. 2006; 59:949–955. discussion 955-946. [PubMed: 17143231]
19. ter Haar GR. High intensity focused ultrasound for the treatment of tumors. *Echocardiography*. 2001; 18:317–322. [PubMed: 11415504]
20. Clement GT. Perspectives in clinical uses of high-intensity focused ultrasound. *Ultrasonics*. 2004; 42:1087–1093. [PubMed: 15234170]
21. Burfeindt MJ, Zastrow E, Hagness SC, Van Veen BD, Medow JE. Microwave beamforming for non-invasive patient-specific hyperthermia treatment of pediatric brain cancer. *Phys. Med. Biol.* 2011; 56:2743–2754. [PubMed: 21464536]
22. Zastrow E, Hagness SC, Van Veen BD, Medow JE. Time-multiplexed beamforming for noninvasive microwave hyperthermia treatment. *IEEE Trans. Biomed. Eng.* 2011; 58:1574–1584. [PubMed: 21216700]
23. Zastrow E, Hagness SC, Van Veen BD. 3D computational study of noninvasive patient-specific microwave hyperthermia treatment of breast cancer. *Phys. Med. Biol.* 2010; 55:3611–3629. [PubMed: 20526033]
24. Raoof M, Curley SA. Non-invasive radiofrequency-induced targeted hyperthermia for the treatment of hepatocellular carcinoma. *Int. J. Hepatol.* 2011; 2011:676957. [PubMed: 21994866]
25. Ohguri T, Yahara K, Moon SD, Yamaguchi S, Imada H, Terashima H, Korogi Y. Deep regional hyperthermia for the whole thoracic region using 8 MHz radiofrequency-capacitive heating device. relationship between the radiofrequency-output power and the intra-oesophageal temperature and predictive factors for a good heating in 59 patients. *Int. J. Hyperthermia*. 2011; 27:20–26. [PubMed: 20858084]
26. Ohguri T, Imada H, Yahara K, Morioka T, Nakano K, Terashima H, Korogi Y. Radiotherapy with 8-MHz radiofrequency-capacitive regional hyperthermia for stage III non-small-cell lung cancer: the radiofrequency-output power correlates with the intraesophageal temperature and clinical outcomes. *Int. J. Radiat. Oncol. Biol. Phys.* 2009; 73:128–135. [PubMed: 18513887]
27. Gellermann J, Wlodarczyk W, Feussner A, Fahling H, Nadobny J, Hildebrandt B, Felix R, Wust P. Methods and potentials of magnetic resonance imaging for monitoring radiofrequency hyperthermia in a hybrid system. *Int. J. Hyperthermia*. 2005; 21:497–513. [PubMed: 16147436]
28. Massodi I, Raucher D. A thermally responsive Tat-elastin-like polypeptide fusion protein induces membrane leakage, apoptosis, and cell death in human breast cancer cells. *J. Drug Target*. 2007; 15:611–622. [PubMed: 17968715]
29. Maeda H, Seymour LW, Miyamoto Y. Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo. *Bioconjug. Chem.* 1992; 3:351–362. [PubMed: 1420435]

Fig. 1. Heating tumors with infrared light. Tumor temperature (as monitored by a needle thermocouple in the tumor core), body temperature, and skin temperature over the heated site was recorded while illuminating the tumor with 950 nm light from an LED light source. Data represent the mean of three mice bearing 250 mm³ E0771 mammary tumors, bars, s.d.

Fig. 2. Plasma stability, plasma clearance and tumor uptake of Bac-ELP-H1. (A) Stability of the rhodamine label on Bac-ELP-H1-Rho was determined by incubation in fresh mouse plasma at 37 °C, thermal precipitation of the labeled polypeptide, and spectrophotometric detection of released label in the remaining plasma. Bars, s.d. (B) Plasma levels with time following IV or IP injection of Bac-ELP1-H1-Rho. Data represent the mean $\pm$ s.d. of 6 animals per group. (C) Representative images of tumor sections 3 h after IV or IP injection of rhodamine-labeled Bac-ELP1-H1. The perfused vasculature was marked by infusion of high molecular weight dextran 1 min prior to euthanasia (top panel), and rhodamine fluorescence was used to follow the localization of the polypeptide within the tumor (middle panel). (D)

Bac-ELP1-H1-Rho tumor levels 3 h after IV or IP administration of rhodamine-labeled Bac-ELP1-H1 with and without tumor hyperthermia. Bars, s.e. (E) Bac-ELP1-H1-Rho and Bac-ELP2-H1-Rho tumor levels 3 h after IV administration of polypeptides with or without hyperthermia. Bars, s.e. (F) High magnification images of the tumors in C were obtained using a fluorescence microscope and a 40 × objective. Cell nuclei were stained with Hoechst 33342.

Fig. 3. Biodistribution of Bac-ELP-H1 Following IV or IP Injection. Organ distribution of Bac-ELP1-H1-Rho 3 h after IV or IP administration with or without tumor heating. Bars, s.e. *, Organ levels are statistically different, (one-way ANOVA, $p = 0.0006$ (liver), $p = 0.005$, (spleen), post hoc Bonferroni, 95% CI). ** Brain levels were not detectable over background fluorescence following IV injection.

Fig. 4.

In vivo tumor deposition of Bac-ELP1-H1 with time. (A) Representative fluorescence images 8 h after injection with saline (left panel) or AlexaFluor 750-labeled Bac-ELP1-H1 (right panels). A mouse with no tumor (second panel), an unheated tumor (third panel), and a heated tumor (fourth panel) are shown. The position of the tumor or the corresponding non-tumor bearing mammary fat pad are shown by the ovals. (B) Quantitative analysis of the tumor fluorescence at each time point. Data represent the mean $\pm$ s.e. of 5 mice/group. *, The difference between heated and unheated tumors is statistically significant ($p = 0.007$, student's t-test).

Fig. 5. Tumor Volume Reduction by Bac-ELP-H1. E0771 Tumor volume (A and C) and corresponding body weight (B and D) after 7 daily treatments (arrows) with saline, saline + hyperthermia, Bac-ELP1, Bac-ELP1-H1, Bac-ELP1-H1 + hyperthermia (A and B), Bac-ELP2-H1, and Bac-ELP2-H1 + hyperthermia (C and D). Data represent the mean $\pm$ s.d. of 6 mice/group. *, Tumor volumes are statistically significant from control (one-way ANOVA, $p = 0.0017$, post hoc Bonferroni, 95% CI). (E) Tumor mass on day 14. *, Tumor mass is statistically significant from control (one-way ANOVA, $p = 0.0008$, post hoc Bonferroni, 95% CI).

Table 1

Plasma pharmacokinetics of Bac-ELP-H1. Plasma clearance data following IV injection of rhodamine-labeled Bac-ELP1-H1, with or without tumor heating, were fit to a two compartment pharmacokinetic model.

	Bac-ELP1-H1 (tumor unheated)	Bac-ELP1-H1 (tumor heated)	Bac-ELP2-H1 (tumor unheated)	Bac-ELP2-H1 (tumor heated)
Initial concentration	140.2 ± 28.9	95.0 ± 26.7	78.9 ± 7.5	81.7 ± 12.5
Central compartment volume of distribution	11.1 ± 1.7	15.5 ± 5.3	20.2 ± 2.3	23.2 ± 3.1
Plasma clearance	0.16 ± 0.03	0.18 ± 0.05	0.28 ± 0.04	0.32 ± 0.06
Area under curve	9713.5 ± 1393.9	7752.4 ± 1665.6	5786.1 ± 861.5	6104.4 ± 1135.3
Tissue to plasma rate constant	0.025 ± 0.008	0.047 ± 0.019	0.029 ± 0.004	0.026 ± 0.004
Plasma to tissue rate constant	0.021 ± 0.014	0.032 ± 0.014	0.049 ± 0.017	0.034 ± 0.009
Elimination rate constant	0.014 ± 0.003	0.012 ± 0.002	0.014 ± 0.001	0.014 ± 0.002
Distribution half life	14.6 ± 4.5	9.4 ± 3.4	8.4 ± 2.0	10.4 ± 1.9
Terminal half life	102.0 ± 9.2	104.4 ± 15.2	152.7 ± 28.7	136.9 ± 23.0