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A Thermally Targeted Peptide Inhibitor of Symmetrical Dimethylation Inhibits Cancer Cell Proliferation

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Abstract

Targeting splicing machinery components is an underdeveloped strategy for cancer therapy. Uridine-rich small nuclear ribonucleoproteins (UsnRNPs) are essential spliceosome components that recognize splice sites in newly transcribed RNA. The major spliceosomal snRNPs are comprised of UsnRNA bound by a ring of Sm proteins. The survival of motor neuron (SMN) complex provides specificity for binding of Sm proteins to UsnRNAs. Three of the seven proteins that comprise the Sm core possess post-translationally modified C-terminal symmetric dimethylarginine (sDMA) residues which promote binding of these proteins to SMN. Here we describe a peptide inhibitor of sDMA that is capable of interfering with SMN/SmB interaction. The inhibitory peptide was attached to Elastin-like polypeptide, a thermally responsive macromolecular carrier, in order to increase its stability and allow enhancement of its cellular uptake by thermal targeting. The fusion polypeptide inhibited the interaction of SMN/SmB, inhibited proliferation, and induced apoptosis in HeLa cells.

Keywords

Elastin-like Polypeptide; thermal targeting; splicing inhibitor; GRG peptide; symmetrical dimethylation

1. INTRODUCTION

Cancer cells carry out transcription, RNA splicing, and translation at high levels in order to produce the amount of proteins needed to maintain their high proliferative rate. Therefore, targeting these processes will have a more pronounced effect on cancer cells than on other non-cancerous senescent or slowly dividing cells. In addition, since the selection of splice sites can be modified by increases in concentration and/or activity of splicing factors [32], it is possible that aberrantly expressed splicing factors in tumor cells contribute to tumorigenicity by production of mRNA isoforms not present in normal cells ([19], and reviewed in [16]). Targeting mRNA splicing for cancer therapy is a promising approach that could lead to new drugs with cancer-cell specific activity.

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Several examples of splicing inhibitors have shown preclinical success for cancer therapy. Spliceostatin A is a natural product derivative that binds to SF3b, a subcomplex of the U2 small nuclear ribonucleoprotein (snRNP), and inhibits splicing [18]. Spliceostatin A induced cell cycle arrest in HeLa cells by contributing to the accumulation of cyclin dependent kinase inhibitors. Also, pladienolide, a known antitumor macrolide, was shown to bind SF3b and inhibit splicing [22]. Pladienolide B was shown to have activity against 39 cancer cell lines, including drug resistant cell lines, *in vitro* and antitumor activity against 6 human tumor xenografts *in vivo* ([34], and reviewed in [48]). Importantly, 5-fluorouracil, a widely used anti-cancer drug since the 1950s, was recently found to disrupt snRNP biogenesis and impair splicing, providing a new mechanism for 5-fluorouracil-mediated cell death [49]. These results demonstrate that the splicing machinery is a viable target for cancer therapy.

SMN, the survival of motor neurons protein, plays a crucial role in snRNP biogenesis and is mutated in most patients with the neurodegenerative disease spinal muscular atrophy (SMA). Specifically, SMN as part of the SMN complex, ensures an ordered assembly of 7 Sm proteins onto the snRNA component of the nascent snRNP [21]. The binding of SMN to some Sm proteins is enhanced by symmetrical dimethylation of Sm protein arginine residues [10,14] and this modification is catalyzed by protein arginine methyltransferase (PRMT) 5 and 7 [9–11,15,24,36]. Symmetrical dimethylarginine (sDMA) polypeptides are a feature of active spliceosomes, and splicing reactions may be inhibited by hypomethylated nuclear extracts and sDMA-specific antibodies [7]. Additionally, a synthetic sDMA peptide containing arginines in the context of a GRG motif is capable of inhibiting SMN interactions with Sm B/B', D1, D3 and UsnRNPs [10]. In fact, a peptide containing the GRG motif alone is a suitable substrate for sDMA modification [25].

Our lab is interested in developing therapeutic peptides (TP) for cancer therapy. TPs are of particular interest because they are easily designed and produced, and can have unparalleled specificity for their target proteins [6,38]. The clinical limitation of TPs lies in their sensitivity to degradation and poor tumor penetration *in vivo* [26,44]. We attempt to overcome these hurdles by attaching the TP to a polypeptide carrier capable of increasing its plasma half-life and targeting it to the tumor site [37]. This report describes a TP that contains repeats of the GRG motif designed to interfere with UsnRNP biogenesis, and this peptide is attached to a thermally responsive polypeptide carrier.

Elastin-like polypeptide (ELP) is comprised of the repeating pentapeptide sequence VPGXG, where X can be any residue other than proline [46]. Each ELP construct has a unique inverse transition temperature (T_i), above which the polymers aggregate [46,47]. The aggregation of ELP is fully reversible when the temperature is lowered below the T_i , and the polypeptide will become soluble again. The inverse transition temperature of ELP can be calibrated to any desired temperature by adjusting the polarity of the amino acid at the X position and by adjusting the number of VPGXG repeats. The phase transition of ELP can be harnessed for targeted drug delivery by inducing focused hyperthermia at the diseased location. When injected systemically, ELP will remain soluble and eventually be cleared from circulation at normal body temperature, but will aggregate and accumulate at a tumor site where local mild hyperthermia is applied [12,28,33]. The efficacy of using ELP as a thermally targeted drug delivery vector has been demonstrated in previous studies by attaching ELP to a peptide inhibitor of c-Myc [1,2,6], to a cell cycle inhibitory peptide based on p21 [29,30], and to the chemotherapeutic drug doxorubicin [3,4].

In this study, we generated fusion polypeptides by modifying ELP with a cell penetrating peptide (CPP) at the N-terminus and a GRG peptide at the C-terminus (Figure 1a). The CPP used was SynB1, a peptide derived from protegrin and known to mediate cellular uptake of large cargo [13, 42]. The GRG peptide consists of 6 Arg residues placed in the context of the

GRG motif, and is designed to interfere with UsnRNP biogenesis by competing with Sm proteins for methylation by PRMT5 and 7. The SynB1-ELP-GRG polypeptide was capable of entering HeLa cells, localizing to the cytoplasm, inhibiting cell proliferation, and inducing apoptosis, and all effects were enhanced when cellular uptake was increased by hyperthermia treatment. Furthermore, treatment of a stable HeLa cell line expressing YFP-SmB with SynB1-ELP-GRG resulted in decreased SMN/SmB interaction that may be attributed to a global disruption of sDMA-modifications.

2. MATERIALS AND METHODS

2.1 Polypeptides

ELP was modified by addition of the SynB1 peptide (RGGRLSYSRRRFSTSTGR) and the GRG peptide (GRGRGRGRGRGR) as described in [1]. All polypeptides were expressed and purified by inverse transition cycling as described in [1], and the T_i was determined [1]. Polypeptides were labeled on a single cysteine residue with fluorescein or rhodamine as in [1].

2.2 Cell culture and polypeptide treatment

HeLa, *MCF-7*, *MDA-MB-231*, and *MCF-10A* cells (acquired from and screened for contamination by ATCC, Manassas, VA) were cultured as previously described [2] using the ATCC recommended media. *E0771 mouse breast cancer cells were obtained from Dr. F.M. Sirotnak (Memorial Sloan-Kettering Cancer Center, New York, NY) and cultured using RPMI medium supplemented with 10% fetal bovine serum (Gemini Bioproducts), 1% antibiotic/antimycotic solution (Mediatech Cellgro), and 2 g/L NaHCO₃.* For experiments, cells were plated in 6-well plates (300,000 cells per well) for flow cytometry, on 22 mm² coverslips for confocal and DIC microscopy, and in 24-well plates (5,000 cells/well) for proliferation assays. Cells were treated with polypeptides dissolved in medium for one hour at either 37 °C or 42 °C, rinsed with sterile phosphate buffered saline (PBS), and replaced with fresh medium and kept at standard culture conditions until analysis. The HeLa cell line stably expressing YFP-SmB was obtained from Dr. Angus Lamond (University of Dundee, Dundee, Scotland). YFP-SmB cells were cultured and harvested as described above for HeLa cells.

2.3 Flow cytometry

2.3.1 Cellular uptake—24 h after polypeptide exposure (20 μM), cells were collected using non-enzymatic cell dissociation buffer, rinsed with PBS, and the mean fluorescence intensity was determined by flow cytometry. Forward versus side scatter gating was used to remove cell debris from the analysis, and fluorescein fluorescence intensity (n = 5,000 cells) was measured using a Cytomics FC 500 flow cytometer and CPX software (Beckman Coulter). Fluorescence intensity was corrected for polypeptide labeling efficiency and normalized to cellular autofluorescence. Data represent the mean ± s.e.m. of 3 independent experiments.

2.3.2 Annexin-V binding—72 h after treatment with the indicated polypeptides (40 μM), both floating and attached cells were harvested and stained with FITC conjugated annexin-V and propidium iodide (PI) as per the manufacturer's instructions (Molecular Probes). The cells (n = 5,000) were then analyzed using a Cytomics FC 500 flow cytometer. Forward versus side scatter gating was used to eliminate cell debris from the analysis, and a scatter plot of PI intensity vs FITC intensity was used to score healthy, apoptotic, and necrotic cells. The percentage of annexin-V positive, apoptotic cells was expressed as an average of 3 experiments; error bars, ± s.e.m.

2.4 Microscopy

2.4.1 Confocal microscopy—HeLa cells growing on 22 mm² coverslips were treated with rhodamine labeled SynB1-ELP1-GRG (20 μM) as described above. 24 h after treatment, cells were fixed with 4% paraformaldehyde, mounted onto slides, and visualized using a Leica TCS SP2 laser scanning confocal microscope (Leica).

2.4.2 Differential Interference Contrast (DIC) microscopy—HeLa cells growing on 22 mm² coverslips were treated with the indicated polypeptide (40 μM) as described above. 72 hours after treatment, the coverslips were mounted onto slides and immediately photographed (without fixation) using DIC microscopy with an Axiovert 200 inverted microscope equipped with a 40x objective (Zeiss) and a CoolSnap (Roper Scientific) CCD camera.

2.5 Cell proliferation

72 hours after a 1 h polypeptide exposure as described above, cells were rinsed to remove dead cells and debris, and attached cells were harvested and counted using a Coulter Counter (Beckman Coulter). All data presented represents the mean ± s.e.m. of at least three independent experiments.

2.6 Antibodies, co-immunoprecipitation, and western blotting

Cells were treated as described above and lysed by resuspension in mRIPA buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA and 1% v/v NP-40), followed by centrifugation to pellet cell debris. Immunoprecipitation reactions were conducted as previously described [43], except that 1 μg of polyclonal antibodies to SMN (H-195, Santa Cruz Biotechnology) was added to the pre-cleared supernatants. SDS-PAGE and western blotting were done as previously described [43]. YFP-SmB was detected using a monoclonal GFP antibody (mixture of clones 7.1 and 13.1, Roche). SMN was detected using a monoclonal SMN antibody (clone 8, BD Biosciences). Western blot chemiluminescent signals were captured and band intensities quantified using the Molecular Imager ChemiDoc XRS+ System and Quantity One 1-D analysis software (Bio-Rad). Values of YFP-SmB/SMN for each treatment condition were normalized to values obtained for untreated cells, (YFP-SmB/SMN)/Untreated. Data show the mean ± s.d. of a minimum of three separate experiments.

2.7 Statistical Analysis

Differences among untreated control, SynB1-ELP control, and SynB1-ELP-GRG treated groups at both treatment temperatures in the polypeptide uptake, cell proliferation, annexin-V binding, and western blotting assays were probed using a one-way ANOVA and a post-hoc Bonferroni multiple comparison with a 95 % confidence interval.

3. RESULTS

3.1 Determination of the T_i of SynB1-ELP-GRG polypeptides

SynB1-ELP-GRG polypeptides were constructed using either the ELP1 polypeptide, which has a T_i just above body temperature and is useful for thermal targeting, or the ELP2 polypeptide, which has a T_i much higher than body temperature, does not aggregate at the 42 °C hyperthermia temperature used in this study, and serves as a control to parse the effects of ELP aggregation from the non-specific effects of hyperthermia. Since modifications to the parent ELP polypeptide (such as addition of the SynB1 CPP and the GRG peptide) and solution conditions (concentration of salt and other dissolved proteins) can alter the T_i, we determined the T_i of SynB1-ELP1-GRG and SynB1-ELP2-GRG under the conditions used for cell culture treatment. As shown in Figure 1b, at 40 μM (the highest concentration used for cell treatment),

SynB1-ELP1-GRG had a T_t of 40 °C, and at 42 °C, the polypeptides were nearly all present in aggregated form. In contrast, SynB1-ELP2-GRG had a T_t of 65 °C. Another factor that influences the T_t of ELP-based polypeptides is the concentration of the polypeptide itself. In order to determine the concentration range in which the T_t lies in the desired window was determined for solutions of various concentrations of SynB1-ELP1-GRG between 37 °C and 42 °C, the T_t in cell culture media. As shown in Figure 1c, as the concentration of the polypeptide decreases, the T_t increases. At 40 μ M SynB1-ELP1-GRG, the T_t is 40 °C, ideal for thermal targeting. At 20 μ M, SynB1-ELP1-GRG had completed about 50% of its transition at 42 °C. At concentrations below 20 μ M, only a small amount of the polypeptides were present as aggregates. Therefore, the effects of thermal targeting by SynB1-ELP1-GRG will be greatest at concentrations above 20 μ M.

3.2 Cellular uptake and subcellular localization of SynB1-ELP-GRG polypeptides

In order to determine if the aggregation of the ELP polypeptide causes increased cellular uptake, the thermally responsive SynB1-ELP1-GRG polypeptide and the non-thermally responsive control SynB1-ELP2-GRG polypeptide were labeled with fluorescein. 24 h after exposure of HeLa cells to a solution of each labeled polypeptide for 1 h at 37 °C or 42 °C, the amount of polypeptide inside and associated with the cell surface was determined by flow cytometric analysis of the cells' mean fluorescence intensity. As shown in Figure 2, some polypeptide accumulated on or in the cells at 37 °C, to levels from 2 to 20 fold above autofluorescence. When the polypeptide incubation was carried out at 42 °C, SynB1-ELP1-GRG accumulated on or in cells at over 17 – fold higher levels than were achieved by incubation with the same polypeptide at 37 °C (1 way ANOVA, $p = 3 \times 10^{-9}$; Bonferroni, 95% CI). In contrast, no difference was seen between cellular levels of SynB1-ELP2-GRG following exposure at 37 °C or 42 °C, and SynB1-ELP2-GRG levels (at either temperature) were not significantly different from SynB1-ELP1-GRG levels at 37 °C. These results indicate that hyperthermia can greatly increase the amount of the ELP1-containing polypeptide taken up by or associated with the cells. And, the increase is due to uptake/association of polypeptide aggregates with the cells, not to non-specific effects of hyperthermia.

The flow cytometry assay is useful for accurate quantitation of cellular polypeptide levels, but it can not distinguish intracellular polypeptide from polypeptide associated with the outside of the cell membrane. To insure that the SynB1-ELP1-GRG polypeptide is internalized by the cells and to determine its subcellular localization, confocal microscopy was carried out using the same treatment conditions as for flow cytometry. The subcellular localization of rhodamine-labeled SynB1-ELP1-GRG was determined using Sytox green as a co-stain and nuclear marker. As shown in Figure 2b, 24 h after 37 °C exposure, SynB1-ELP1-GRG-Rho was visible in the cytoplasm as punctate staining, and was mostly excluded from the nucleus. Larger aggregates of the polypeptide were present in the cytoplasm after 42 °C treatment, and images were brighter, reflecting the higher levels of intracellular polypeptide. These data confirm that the SynB1 CPP mediates internalization of the polypeptide, and they demonstrate that the polypeptide adopts a primarily cytoplasmic distribution. Since SMN complex-mediated assembly of Sm proteins onto snRNA in snRNP biogenesis takes place in the cytoplasm, it is important for the SynB1-ELP-GRG polypeptide to localize in this cellular compartment in order to disrupt SMN/Sm protein interaction.

3.3 Inhibition of cell proliferation by SynB1-ELP-GRG polypeptides

To examine the anti-proliferative effects of these polypeptides, HeLa cells were exposed to the polypeptides for 1 hour at 37 °C or 42 °C as described in the Methods. Cell number was determined 72 h after treatment using a Coulter Counter. As shown in Figure 3a, exposure of cells to SynB1-ELP1-GRG (40 μ M) at 37 °C inhibited cell proliferation by 25%, and the antiproliferative effect was increased 2 – fold when the polypeptide treatment was conducted

at 42 °C (1 way ANOVA, $p = 4.9 \times 10^{-10}$; Bonferroni, 95% CI). Exposure to hyperthermia alone had no significant effect on cell proliferation. Also, the SynB1-ELP1 control polypeptide which lacks the GRG peptide had no effect on cell proliferation. SynB1-ELP2-GRG inhibited proliferation by 25 %, similar to SynB1-ELP1-GRG without hyperthermia, and the effect of the ELP-2-containing polypeptide was not enhanced by hyperthermia treatment. This observation demonstrates that the 2 – fold enhancement of potency seen for SynB1-ELP1-GRG with hyperthermia was due to aggregation and increased uptake of the polypeptide, not to nonspecific effects of hyperthermia. As shown in Figure 3b, the antiproliferative effect of SynB1-ELP1-GRG was concentration dependent, and the potency was significantly enhanced by hyperthermia treatment at all concentrations tested (1 way ANOVA, $p = 1.9 \times 10^{-9}$; Bonferroni, 95% CI). Consistent with the single dose findings shown in Figure 3a, the SynB1-ELP1 control polypeptide had no effect on cell proliferation at any concentration tested.

3.4 Induction of apoptosis by SynB1-ELP-GRG polypeptides

In order to evaluate the mechanism of cell death, the cells were treated with control (medium-only) or 40 μ M SynB1-ELP1, SynB1-ELP1-GRG, or SynB1-ELP2-GRG with and without hyperthermia. 72 hours following treatment, the cells were harvested and stained with FITC conjugated Annexin-V and PI to mark apoptotic and necrotic cells, respectively, and analyzed by flow cytometry. Figure 4a shows representative scatter plots of the flow cytometric data collected for SynB1-ELP1 (top panel) and SynB1-ELP1-GRG (bottom panel) following 37 °C (left panel) or 42 °C (right panel) exposure. Less than 2 % of the analyzed cells were Annexin-V positive following SynB1-ELP1 treatment at either temperature. Likewise, no apoptosis was evident following SynB1-ELP1-GRG treatment at 37 °C. However, 72 h after treatment with SynB1-ELP1-GRG at 42 °C, 17 % of the analyzed cells were stained by Annexin-V, indicating that the GRG peptide induces apoptosis in these cells. There was no evidence of necrosis under any of the treatment conditions. The percentage of apoptotic cells (quadrant K1 from figure 4a) was averaged for 3 independent experiments and is shown in Figure 4b. After treatment with SynB1-ELP1-GRG at 42°C, there was a 3 – 4 fold increase in the number of apoptotic cells as compared to the 37 °C treatment (1 way ANOVA, $p = 1.2 \times 10^{-6}$; Bonferroni, 95% CI). Furthermore, no apoptosis was observed following treatment with the polypeptide carrier alone, SynB1-ELP1, at 37 °C or 42 °C, or with hyperthermia alone. When treated with the SynB1-ELP2-GRG non-thermally responsive control polypeptide, HeLa cells showed no evidence of apoptosis at either temperature.

The induction of apoptosis by SynB1-ELP1-GRG was also confirmed by assessing the cell morphology after polypeptide treatment. HeLa cells growing on glass coverslips were treated as described above. 72 h after treatment, the cell morphology was observed using DIC microscopy (Figure 4c). Control treated and SynB1-ELP1 treated cells were spread normally onto the substratum and displayed intact nuclei. Hyperthermia treatment had no effect on the cell morphology following medium-only control or SynB1-ELP1 treatment. Cells treated with SynB1-ELP1-GRG at 37 °C were also morphologically indistinguishable from control treated cells. In contrast, following treatment with SynB1-ELP1-GRG at 42 °C, many cells were rounded and displayed membrane blebbing and loss of nuclear structure, all hallmarks of apoptosis.

3.5 Treatment with SynB1-ELP-GRG reduces SMN and YFP-SmB interaction

The SMN/SmB protein – protein interaction is enhanced by sDMA. To address the effects of GRG-containing polypeptide treatment on the SMN/SmB interaction, lysates were obtained from a HeLa cell line stably expressing YFP-SmB. YFP-SmB cells were untreated, or treated with 20, 30 or 40 μ M SynB1-ELP1-GRG or SynB1-ELP1 control for 1 h at 42 °C. 24 h after treatment, cell lysates were subjected to immunoprecipitation with polyclonal anti-SMN antibodies followed by western blotting with anti-GFP and anti-SMN monoclonal antibodies

(Figure 5). Treatment of cells with SynB1-ELP1-GRG decreased the amount of YFP-SmB co-immunoprecipitating with SMN (lanes 3, 4, and 5) compared to cells treated with SynB1-ELP1 control (lanes 6, 7, and 8), or untreated (lane 2) (Figure 5a). No YFP-SmB was recovered when immunoprecipitation was performed using normal rabbit serum only (lane 1). Data was averaged from a minimum of three independently performed experiments. Relative recoveries of YFP-SmB were found to be significantly reduced at all concentrations tested following SynB1-ELP1-GRG treatment compared to that obtained with SynB1-ELP1 treatment (1 way ANOVA, $p = 1 \times 10^{-4}$, Bonferroni, 95% CI) and relative to untreated cells (Figure 5b). These results demonstrate that the GRG peptide may induce apoptosis in HeLa cells by inhibiting the interaction of SMN with SmB and presumably other sDMA proteins known to interact with SMN.

3.6 Antiproliferative effects of SynB1-ELP-GRG in breast cancer and non-tumorigenic breast epithelial cell lines

In order to determine whether the antiproliferative effects of the SynB1-ELP-GRG polypeptide were specific to HeLa cells, we repeated the cell proliferation assay using a number of breast cancer cell lines and a non-tumorigenic breast epithelial cell line. As shown in Figure 6, all cell lines showed some concentration-dependent response to SynB1-ELP1-GRG treatment. The human breast cancer cell lines MCF-7 (Figure 6A) and MDA-MB-231 (Figure 6B) showed similar sensitivity to the polypeptide treatment, with a maximum of 55–60% proliferation inhibition induced by 40 μ M polypeptide at the hyperthermia temperature. The mouse breast cancer cell line E0771 was even more sensitive to SynB1-ELP1-GRG, showing a maximum inhibition of nearly 80%, and the antiproliferative effect was significantly enhanced by hyperthermia in this cell line (Figure 6C). The non-tumorigenic human breast epithelial cell line MCF-10A was least affected by SynB1-ELP1-GRG treatment (Figure 6D). Treatment at 37 °C had no effect at concentrations up to 20 μ M, and only approximately 30% inhibition at 40 μ M. When treatment was combined with hyperthermia, there was a slight enhancement in the polypeptide's potency, but the maximum inhibition of approximately 50% was less than that observed with the cancer cell lines.

4. DISCUSSION

In this study, in order to disrupt SMN function, fusion polypeptides consisting of a cell penetrating peptide, an ELP, and a GRG peptide were generated, and the thermal properties of the ELP were exploited to increase cellular uptake of the GRG peptide. The SynB1-ELP1-GRG polypeptide inhibited cell proliferation and induced apoptosis in HeLa cells, and both effects were enhanced by combining polypeptide treatment with hyperthermia. The antiproliferative effect was also observed in several breast cancer cell lines, but the polypeptide was less potent in a non-tumorigenic breast epithelial cell line. The SynB1-ELP1-GRG polypeptide was shown to reduce SMN/SmB interaction, which would result in reduced UsnRNP formation and splicing.

Considering the important role that SMN plays in the formation of part of the machinery necessary for RNA splicing, a fundamental cellular process, targeting the SMN complex as a therapeutic avenue for cancer is logical. Since mutations in SMN result in SMA and motor neuron cell death, localized disruption of the SMN complex in tumor cells via thermal targeting could provide a heretofore unexplored strategy for cancer therapy. Recent work showing that the well established anti-cancer agent 5-fluorouracil can alter snRNA modifications and reduce splicing [49] further validates the need to develop cancer therapies that target snRNP biogenesis.

Previous studies have shown that synthetic peptides are capable of inhibiting SMN interactions with Sm B/B', D1, D3, and UsnRNPs [10], and the GRG motif alone is adequate for sDMA

to occur [25]. Therefore, the GRG-containing polypeptides utilized in this study may undergo sDMA modifications through interactions with PRMTs that are capable of symmetrically dimethylating Sm proteins. We hypothesize that this extra influx of sDMA-modified peptide may decrease the pool of functional SMN by forming dead-end SynB1-ELP1-GRG/SMN complexes. In support of this hypothesis, we observed a reduction in SMN/SmB interaction upon SynB1-ELP1-GRG treatment with hyperthermia (Figure 5). Although not tested here, we would expect that SynB1-ELP1-GRG treatment would also decrease the interaction of SMN with other Sm proteins known to have sDMAs (SmB', D1 and D3). Alternatively, our results do not rule out the possibility that SynB1-ELP1-GRG treatment globally disrupts sDMA modifications, impacting vital cellular functions beyond snRNP biogenesis and splicing. Over 200 proteins are known to contain sDMA, although few of these have been identified [8]. Other sDMA-modified proteins play major roles in cell growth, proliferation, and regulation of a variety of disorders [11,20,23,35,39–41,45]. Notable among these, PRMT5 has been shown to function as a complex co-factor that interacts with and methylates p53, thus affecting its target gene specificity, and depletion of PRMT5 results in p53-dependent apoptosis [17]. Although our initial approach was designed to decrease snRNP biogenesis by reducing Sm protein interaction with SMN, disruption of PRMT5/7 activity using SynB1-ELP1-GRG is expected to have wide-ranging consequences which are deleterious for cancer cell proliferation.

5. CONCLUSIONS

The results presented here demonstrate that the GRG peptide is potentially a promising new tool for cancer therapy. Fusing the GRG peptide to the ELP carrier is advantageous because ELP has a long half-life *in vivo* [27] and can be targeted to the site of a tumor by externally applied hyperthermia [12,28,33]. The utility of ELP for delivery of previously validated therapeutic peptides has been demonstrated by our lab [1,2,5,29–31], and this work extends the previous studies by utilizing the ELP technology for delivery of a peptide inhibitor of symmetrical dimethylation. This work describes a novel therapeutic peptide delivered using innovative polypeptide engineering strategies, and it validates symmetrically dimethylated proteins as new targets for cancer therapy.

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Abbreviations

CPP	cell penetrating peptide
ELP	elastin like polypeptide
GRG	peptide inhibitor of arginine methylation
PRMT	protein arginine methyltransferase
sDMA	symmetrical dimethylarginine
Sm	family of proteins that form a ring in the snRNP complex
SMA	spinal muscular atrophy
SMN	survival of motor neuron protein

snRNPs	small nuclear ribonuclear proteins
<i>SynBI</i>	protegrin-derived cell penetrating peptide
TP	therapeutic peptide
T _t	transition temperature

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Figure 1.

Design and thermal properties of SynB1-ELP-GRG polypeptides. **(A)** The ELP polypeptide carrier was modified by addition of the SynB1 CPP at the N – terminus and the GRG TP at the C – terminus. **(B)** The T_t of SynB1-ELP1-GRG and SynB1-ELP2-GRG was determined for 40 μ M polypeptide in cell culture medium. SynB1-ELP1-GRG had a T_t ideal for thermal targeting under these conditions, and SynB1-ELP2-GRG had a T_t much higher than the hyperthermia temperature of 42 °C, making it a suitable control for parsing the effects of ELP aggregation from the effects of hyperthermia. **(C)** The concentration dependence of the SynB1-ELP1-GRG T_t was determined. Absorbance data were converted to percentage of the

maximum OD₃₅₀ to allow comparison of all polypeptide concentrations on the same plot. Concentrations of 20 μ M and above were found to be optimal for thermal targeting.

Figure 2. Cellular uptake and subcellular localization of SynB1-ELP-GRG polypeptides. **(A)** The uptake and/or association of polypeptides with the cells were determined 24 h after a 1 h exposure using flow cytometry. The cellular uptake/association of SynB1-ELP1-GRG increased 17 – fold when polypeptide treatment was combined with hyperthermia, while the uptake/association of SynB1-ELP2-GRG was unaffected by hyperthermia treatment. * Cellular uptake/association is significantly increased relative to SynB1-ELP1-GRG at 37 °C (1 – way ANOVA, post – hoc Bonferroni). **(B)** The subcellular distribution of SynB1-ELP1-GRG was determined 24 h after a 1 h exposure to the polypeptide by confocal fluorescence microscopy. Sytox green (left panel) was used as nuclear stain for reference, and polypeptide localization was detected using rhodamine – labeled polypeptide (middle panel). SynB1-ELP1-GRG was

found to localize to the cytoplasm, and large polypeptide aggregates were seen in the cells following exposure at 42 °C. Scale bar = 20 μ m.

Figure 3.

SynB1-ELP-GRG polypeptides inhibit HeLa cell proliferation. **(A)** Proliferation of HeLa cells was determined 72 h after a 1 h exposure to the indicated polypeptide at 37 °C or 42 °C. The antiproliferative effect of the thermally responsive polypeptide SynB1-ELP1-GRG was increased 2 – fold by hyperthermia treatment, and all proliferation inhibition was attributable to the GRG peptide. **(B)** The concentration dependence of the antiproliferative effect of SynB1-ELP1-GRG was determined using the same assay. Hyperthermia enhanced the potency of SynB1-ELP1-GRG at all concentrations tested, but most notably at 20 μM and 40 μM. No inhibition of proliferation was observed following treatment with SynB1-ELP1. * Cell proliferation is significantly inhibited relative to untreated (37 °C) cells. † The difference in cell proliferation between treatment at 37 °C and 42 °C with the indicated polypeptide is statistically significant (1 – way ANOVA, post – hoc Bonferroni).

Figure 4. SynB1-ELP1-GRG polypeptides induce cell death by apoptosis. **(A)** The mechanism of cell death was determined 72 h after a 1 h exposure to the indicated polypeptide (40 μ M) by staining cells with annexin-V-FITC to mark apoptotic cells and PI to mark necrotic cells. Very few apoptotic cells were observed following SynB1-ELP treatment at both temperatures, or following SynB1-ELP1-GRG treatment at 37 °C. However, a significant proportion of the cell population was apoptotic following exposure to SynB1-ELP1-GRG at 42 °C. **(B)** The percentage of apoptotic cells was averaged from three independent experiments. Induction of apoptosis was significantly enhanced by treatment with SynB1-ELP1-GRG at 42 °C; but SynB1-ELP2-GRG, SynB1-ELP1, or hyperthermia alone had no effect. * Induction of apoptosis is significantly enhanced relative to untreated (37 °C) cells. † The difference in percentage of apoptotic cells between treatment at 37 °C and 42 °C with the indicated polypeptide is statistically significant (1 – way ANOVA, post – hoc Bonferroni). **(C)** Induction of apoptosis was also confirmed by examining the cell morphology by DIC microscopy. Many cells treated with SynB1-ELP1-GRG at 42 °C were rounded with fragmented chromatin and membrane blebs, while all control cells were spread onto the substrate and had intact nuclei.

Figure 5. SynB1-ELP1-GRG inhibits the SMN/SmB interaction. **(A)** Cells from a HeLa cell line stably expressing YFP-SmB were treated with 20, 30 or 40 μM GRG or control peptides for 1 hr at 42 °C as described in the Methods, then subjected to immunoprecipitation with anti-SMN antibodies followed by western blotting with anti-GFP and anti-SMN antibodies. U, untreated cells; NRS, normal rabbit serum; SynB1-ELP1-GRG, cells treated with SynB1-ELP1-GRG; SynB1-ELP1, cells treated with SynB1-ELP1; Input, lysate from untreated cells prior to immunoprecipitation. The bands detected for YFP-SmB (α-GFP) and SMN (α-SMN) are shown. SynB1-ELP1-GRG treatment reduces SMN/SmB interaction as evidenced by a decreased recovery of YFP-SmB from the SMN immunoprecipitations, whereas treatment with the control polypeptide SynB1-ELP1 had no effect. **(B)** Analysis of the YFP-SmB/SMN interaction for cells treated with SynB1-ELP1-GRG or SynB1-ELP1 at different concentrations relative to untreated cells reveals that less YFP-SmB is co-immunoprecipitated from cells treated with increasing concentrations of SynB1-ELP1-GRG. * (YFP-SmB/SMN)/Untreated is significantly inhibited relative to untreated cells (1 – way ANOVA, post – hoc Bonferroni).

Figure 6. Antiproliferative effects of SynB1-ELP-GRG in breast cancer and breast epithelial cell lines. The human breast cancer cell lines MCF-7 (A) and MDA-MB-231 (B), mouse breast cancer cell line E0771 (C), and the human non-tumorigenic breast epithelial cell line MCF-10A (D) were exposed to the indicated concentration of SynB1-ELP1-GRG for 1 h at 37 or 42 °C. 72 h after the initial exposure, the cell number was determined by Coulter counting. Data represent the mean $\pm$ s.e. of 3 to 4 experiments.