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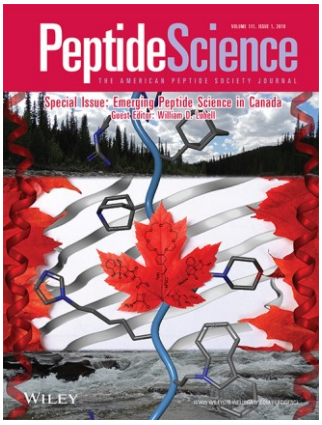
Special Issue: Emerging Peptide Science in Canada

Guest Editor: William D. Lubell, Département de chimie, Université de Montréal



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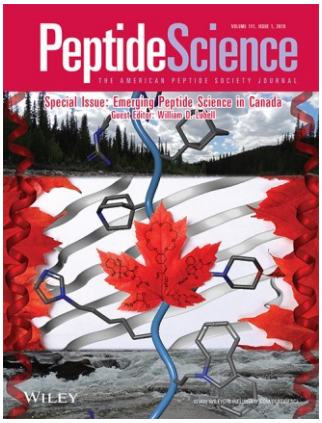

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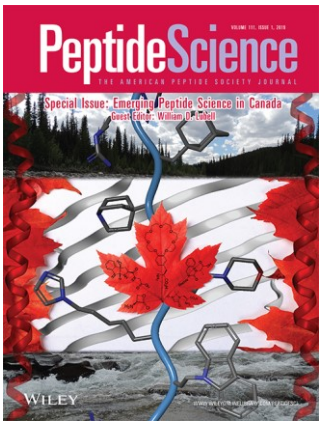

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Received: 30 November 2017 | Revised: 16 January 2018 | Accepted: 18 January 2018

DOI: 10.1002/pep2.24048

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REVIEW

Peptide therapeutics that directly target transcription factors

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Funding information
NSERC Discovery Grant and the Collaborative Health Research Program (CHRP)

Abstract

Transcription factors regulate gene expression in cells and control cellular development, function, and death. Dysregulation of transcription factors is often associated with disease, including cancer. As such, transcription factors are attractive targets for design of therapeutics against disease. Transcription factors function using protein-protein and protein-DNA interactions that occur over relatively large surface areas: this lack of a small and defined "ligand binding site" has proven to be challenging to target with small molecules. Peptide therapeutics, therefore, provide an alternate approach toward design of inhibitory agents. Transcription factors are conveniently modular by design: just the small domain that is responsible for the transcription factor's DNA binding or a protein-protein interaction or another function, can serve as the basis for novel peptide therapeutics. In this review, examples of peptides that *directly* interfere with transcription factors will be discussed.

KEYWORDS
peptide drugs, peptidomimetics, protein design, transcription factors

1 | INTRODUCTION

Transcription factors are proteins that regulate gene expression in cells. These proteins recognize and bind promoters, the specific DNA sequences that lie near the coding region of the gene. This dynamic process of protein-DNA recognition governs a cell's development, function, and ultimately, its death. Once bound to the promoter, the transcription factor typically activates or represses transcription by recruiting or blocking RNA polymerase, respectively. Unlike other types of proteins such as enzymes or receptors, transcription factors are structurally modular; for example, the DNA-binding domain is distinct from the transcriptional activation domain, and the DNA-binding domain from a transcription factor can alone constitute a protein that retains the structure and function of this specific domain. This modularity is readily exploited in the laboratory, as researchers tackle these smaller, more manageable domains in efforts to elucidate structure and function of the much larger transcription factors [see Refs. (1,2) for classic reviews of transcriptions factors and their interactions with DNA].

Because transcription factors play central roles in the cell, mutations in these proteins can lead to aberrant gene regulation, which in turn, can lead to development of diseases [recently reviewed in Ref. (3). Many transcription factors regulate cell survival and proliferation, and their dysregulation is often key to oncogenesis: for example, the MYC transcription factor is involved in numerous normal cellular processes, but in deregulated and overexpressed form, MYC is involved in over half of all human cancers [see Ref. (4) for a recent review of the MYC proto-oncogene]. As such, transcription factors could be considered prime targets for drug development. However, transcription factors have been historically difficult to target, owing in part to the fact that they typically exert their activity via protein-protein and/or protein-DNA interactions. In comparison with proteins such as enzymes that possess a defined ligand-binding site that a small molecule can target, the extensive surface areas of these transcription-factor interactions are difficult to disrupt by using popular approaches such as small-molecule library screening.

Toward filling this gap in drug development, research in peptide therapeutic drugs has steadily increased.[5,6] In particular, this approach is a promising alternative when attempting to target transcription factors. Proteins that naturally partner with transcription factors have evolved to recognize their specific partner's surface: we can borrow this design concept to develop protein inhibitors. For example, peptide therapeutics can competitively interfere with the protein-protein and protein-DNA recognition events that a transcription factor makes while performing its tasks. Design of a peptide therapeutic will typically follow a multistep approach: (1) understanding the molecular structure of the target transcription factor, (2) identification of the modular domains

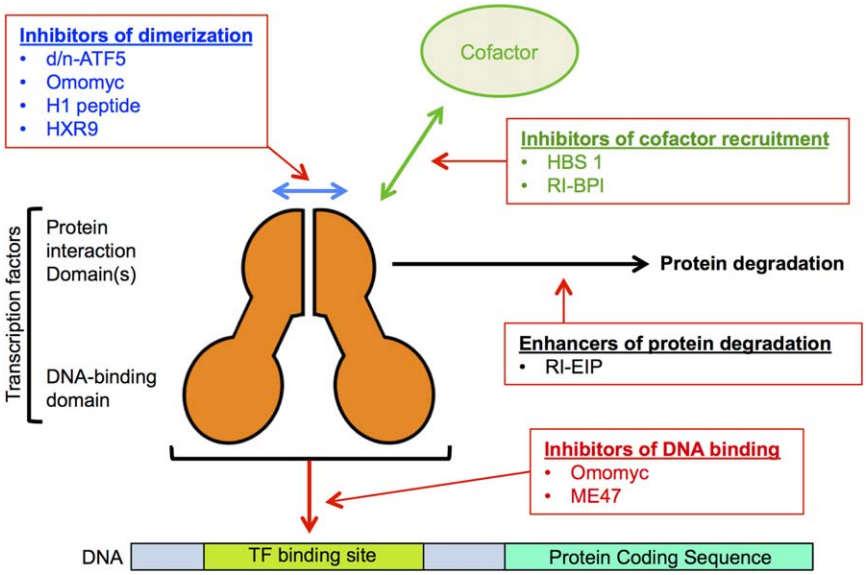

FIGURE 1 Peptide therapeutics inhibit transcription factors (TFs) by interfering with various protein-protein and protein-DNA interactions that are required for TF function. This review discusses peptide inhibitors that affect transcription-factor function by four mechanisms: inhibition of protein-protein dimerization, inhibition of cofactor recruitment, inhibition of protein-DNA recognition, and enhancement of protein degradation

and motifs of the transcription factor that can be targeted, (3) development of a peptide inhibitor that specifically recognizes the targeted transcription-factor domain, and (4) improvement of the peptide inhibitor's properties such as biological activity, ease of delivery, and stability.

In this review, we present examples of peptide therapeutic candidates that directly interact and inhibit their target transcription factors (Figure 1). These examples illustrate both the process behind development of various peptide therapeutics as well as their applications. We have organized our discussion into two major sections: the first section is focused on larger peptide therapeutic candidates comprising 30–100 amino acids (aa), and the second section on shorter peptides comprising 5–20 aa.

2 | LARGE PEPTIDES THAT TARGET TRANSCRIPTION FACTORS

2.1 | ATF5 inhibitors

Activating transcription factor 5 (ATF5) is a member of the activating transcription factor/cyclic-AMP responsive element-binding (ATF/CREB) family. ATF5 expression in neural progenitor stem cells helps maintain the cell line by preventing cellular differentiation.[7] As such, neural progenitor cells express high levels of ATF5, whereas the protein is absent from mature neurons and glia. In 2006, Angelastro et al.[8] observed that ATF5 is expressed in glioblastomas and required for tumor survival and propagation. This suggested that ATF5 is involved in tumor dysregulation, and possibly contributes toward tumor propagation by preventing cell differentiation. ATF5 has also been shown to play roles in other human malignancies, including breast, pancreatic, lung, and colon cancers.[9]

2.1.1 | Designed dominant negative d/n-ATF5

ATF5 is a transcription factor that belongs to the bZIP (basic region/leucine zipper) superfamily of proteins that homodimerize to regulate expression of genes such as antiapoptotic B cell leukemia (Bcl-2) and myeloid cell leukemia-1 (Mcl-1). The bZIP proteins use the basic region for DNA recognition, while the leucine zipper is responsible for dimerization. Angelastro and coworkers designed the dominant negative protein d/n-ATF5 against ATF5 to study its function in human glioma cells.[7] They removed ATF5's N-terminal activation domain and replaced the DNA-binding basic region with a negatively charged amphipathic α-helix containing Leu heptad repeats. The FLAG-tagged version of d/n-ATF5 comprises 100 amino acids and retains the ATF5 leucine zipper that allows d/n-ATF5 to dimerize with ATF5. Upon dimerization of d/n-ATF5 with ATF5, the "basic region" of d/n-ATF5, altered to carry negatively charged residues, interacts with the positively charged ATF5 basic region through electrostatic interactions, thereby inhibiting its DNA-binding function.[7]

Originally, d/n-ATF5 was used to study ATF5 in neural progenitor cells. Attenuation of ATF5 activity in these cells by d/n-ATF5 resulted in increased cellular differentiation, as evinced by upregulation of the neuronal marker, neuron-specific class III β-tubulin, whose presence correlates with differentiation.[7] This study established the importance of ATF5 in maintaining proliferating cells, which prompted the next question: is ATF5 involved in maintaining proliferation of neural cancer cells such as gliomas? Subsequent analysis confirmed ATF5 expression in 29 human glioma cell lines.[8] The activity of d/n-ATF5 was tested in seven of these glioma cell lines. Expression of d/n-ATF5 resulted in apoptotic cell death of up to 40% of the cultured cells, as compared to death of 8% of cells in the negative control.[8] Inhibition of ATF5 using siRNA caused similar levels of apoptotic cell death, suggesting that d/n-ATF5 acts specifically as an ATF5 inhibitor. d/n-ATF5 was then

tested in an animal model. Rats transplanted with the C6 rat glioma cell line were treated with d/n-ATF5, which was delivered to the tumor using a retroviral vector. Three days after d/n-ATF5 delivery, tumor tissue was analyzed by TUNEL staining to monitor apoptotic cell death. Strikingly, nearly all tumor cells (96%) infected with the virus were dead, when compared with death of 1% of control cells.[8] Interestingly, only 2% of normal cells that received d/n-ATF5 showed apoptotic death: d/n-ATF5 did not appear to cause apoptosis in healthy cells.

To further test the efficacy of d/n-ATF5, Arias et al.[10] generated an inducible transgenic mouse model. Here, d/n-ATF5 expression was controlled by two factors: for spatial control, the authors used the human glial fibrillary acidic protein (hGFAP) promoter, which directs protein expression in neural progenitor cells and gliomas; for temporal control, they used the Tet-off system, which regulates timing of induction of protein expression by withdrawing doxycycline from the animals' diet. Glioma development was induced in animals by retroviral delivery of platelet-derived growth factor (PDGF) B.

When doxycycline treatment was withdrawn from the mice—thereby turning on d/n-ATF5 expression—prior to retroviral treatment, only one out of seven animals developed glioma. In contrast, nearly all transgenic mice receiving the retrovirus while continuously given doxycycline developed gliomas within 90–180 days. In addition, expression of d/n-ATF5 after glioma development resulted in the complete regression of tumor; all 24 mice treated in this fashion showed no signs of tumor 40–60 days after doxycycline withdrawal.[10] Tumor regression was confirmed by the behaviour of animals (glioma often causes moribund behavior), as well as through visual and histological analyses. Hence, d/n-ATF5 can prevent both glioma formation and abolish an already established tumor. Strikingly, in control animals, prolonged expression of d/n-ATF5 on the order of months did not appear to affect normal bodily functions.

2.1.2 | Next generation d/n-ATF5 designs

Two parallel studies have recently furthered d/n-ATF5 design. In one study, Angelastro's group removed the C-terminal 25 amino acids of the leucine zipper of d/n-ATF5: this truncated peptide exhibited reduced tendency for aggregation while retaining potency.[11] A 16 aa penetratin domain of the Antennapedia (Antp) homeodomain protein[12,13] was fused to the N-terminus of this truncated d/n-ATF5 to enhance cellular penetration. A 6xHis-tag and a FLAG tag were fused to the peptide to aid purification and immunodetection, respectively. The new protein, Pen-d/n-ATF5-RP, was shown to cross the blood-brain barrier when intraperitoneally delivered to a retrovirally-induced mouse glioma model. Treatment with Pen-d/n-ATF5-RP resulted in complete regression of tumor within 64 h after peptide administration. A long-term follow-up study that used magnetic resonance imaging (MRI) to monitor animals after two administrations of Pen-d/n-ATF5-RP showed that even a year postadministration, the authors observed no signs of tumor ($n = 7$), as well as no adverse side-effects in normal tissues.[11] Similar potency was observed in a mouse xenograft model using the human U87-MG-Luc2 glioma cell line.

Parallel to the study above by Angelastro and coworkers, Karpel-Massler et al.[14] developed CP-d/n-ATF5-S1. This synthetic, 67 aa peptide shares a similar design with Pen-d/n-AFP5-RP, featuring an N-terminal penetratin domain and lacking 25 aa at the C-terminus of the leucine zipper of d/n-ATF5; the major difference between the two peptides is that CP-d/n-ATF5-S1 lacks His and FLAG tags. This peptide was tested in different mouse xenograft models, with each animal representing various cancers such as glioblastoma (U87MG and GBM12 cells), melanoma (A375), prostate cancer (PC3), pancreatic cancer (PANC-1), colorectal cancer (HCT116), and triple-negative breast cancer (MDA-MB-231). In all cases except for PANC-1 and HCT116 cells, treatment by CP-d/n-ATF5-S1 caused a statistically significant attenuation in tumor volume (up to fivefold difference compared with control)[14]; -S1 typically caused a reduction in tumor growth rate, and not tumor regression. However, CP-d/n-ATF5-S1 did cause tumor regression in mouse models bearing the breast cancer cell line MDA-MB-231. No side effects were observed in other healthy tissues, such as the brain, lung, kidney, heart, liver, spleen, and intestine. In addition, in a cell culture study, CP-d/n-ATF5-S1 decreased cell viability of canine glioma cell lines, J3TBg and SDT3G, in a dose-dependent manner.[15] CP-d/n-ATF5-S1 is being explored as a viable anti-cancer treatment not only for humans, but across multiple mammalian species.

Additionally, in a cell culture study, treatment by CP-d/n-ATF5-S1 caused the down-regulation of Bag3 (Bcl-2-associated anthanogene 3) and Usp9X (ubiquitin-specific peptidase 9, X-linked) proteins.[14] Both Bag3 and Usp9X are stabilizers of the antiapoptotic Mcl-1 gene: their downregulation provides hints towards understanding the mode of action for these peptides.

2.2 | MYC inhibitors

MYC is a transcription-factor regulating gene involved in cellular processes such as cell proliferation and differentiation; aberrant expression of MYC is associated with over 50% of all human cancers [see Refs. (16,17) for reviews of MYC-associated cancers]. The MYC protein belongs to the bHLHZ (basic region/helix-loop-helix/leucine zipper) superfamily of transcriptional activators that regulate gene expression by binding to promoter regions as dimers. The MYC basic region binds to the DNA target known as the E-box site (Enhancer box); the consensus E-box sequence is CANNTG, with the palindromic CACGTG serving as the canonical sequence.[18–20] MYC itself does not homodimerize and does not bind to the E-box as a monomer. Instead, MYC uses the HLHZ region to heterodimerize with its partner protein MAX.[21] The MYC/MAX heterodimer is capable of high affinity and sequence-specific binding to the E-box, thereby allowing MYC's activation domain to regulate transcription. In the bHLHZ superfamily, MAX serves as the master regulator by partnering with bHLHZ proteins, including MYC, MXI, and MNT, thereby regulating their function.[22,23] MAX can also homodimerize and bind to the E-box, but there is no known physiological role for the MAX homodimer.

2.2.1 | Omomyc

The first peptide inhibitor reported for MYC, named Omomyc, was reported by Soucek et al. Omomyc is a 92 aa, dominant-negative version of MYC that was generated by making four amino-acid

substitutions in the leucine zipper of the MYC bHLHZ domain. These mutations alter the dimerization specificity of Omomyc by allowing it to form stable homodimers, as well as stable heterodimers with MYC and MAX.[24] Omomyc arrests MYC-dependent proliferation of NIH3T3 fibroblast cells in cell culture. This observation, along with other early studies, pointed towards the therapeutic potential of Omomyc.[24,25]

The efficacy of Omomyc as a cancer therapeutic was shown in a mouse lung adenocarcinoma model; tumors can be generated in these mice by exposing them to aerosolized recombinant adenovirus.[26] A doxycycline-inducible Omomyc gene cassette (TET-on system) was incorporated into the animals' genome, and the cassette induced systemic expression of Omomyc when the mice were fed doxycycline. These mice were exposed to adenovirus and kept for 18 weeks to allow tumor development before Omomyc expression was turned on. The induction of Omomyc expression caused tumor regression within three days, and no visible tumors were detected after four weeks. Importantly, Omomyc regressed the tumors in the mouse adenocarcinoma model without causing any irreversible side effects.[26]

Researchers originally feared that systemic administration of an anti-MYC drug would cause harm by impeding essential MYC activities in normal cells, in addition to controlling tumor growth. Despite these concerns, Omomyc treatment in the aforementioned mouse adenocarcinoma model caused no changes in body weight or blood chemistry.[26] Tissues that normally exhibit high turnover rates, such as the intestine and bone marrow, were affected by Omomyc, but the effect was reversible. In a follow-up study, Soucek et al.[27] investigated the long-term effect of Omomyc exposure using the same mouse model. Here, tumor-induced mice underwent alternating periods where Omomyc expression was turned on and off at regular time intervals. Intriguingly, these animals survived over a year while the treatment completely contained tumor progression. Even after a year, tumors failed to develop resistance mechanisms against Omomyc. Other studies have also shown the potency of Omomyc in both adenocarcinomas and gliomas.[28,29]

Omomyc's mechanism of action has been elusive. Initially, Omomyc was thought to be a dominant-negative repressor of MYC, sequestering aberrantly expressed MYC in an Omomyc/MYC heterodimer—hence, a protein/protein interaction—that thereby decreased the presence of MYC/MAX heterodimer capable of transcriptional activation from E-box sites.[24] However, Eilers and coworkers recently showed Omomyc binding to E-box sites in immunoprecipitation assays.[30] They also used electrophoretic mobility shift assay (EMSA) to quantify comparable binding affinities of the Omomyc/Omomyc homodimer, and Omomyc/MYC and MYC/MAX heterodimers to the E-box target. Mutations in the Omomyc basic region that attenuate its DNA-binding capability also dampened its potency as an anti-MYC agent, suggesting that a protein-DNA interaction is at least partially responsible for Omomyc's anti-MYC function. Therefore, Omomyc may function in two ways: (1) the Omomyc homodimer may competitively inhibit binding to the E-box DNA site by the MYC/MAX transcription factor and (2) Omomyc may sequester excess MYC by heterodimerization.

2.2.2 | ME47

Originally termed "MAXE47,"[31,32] ME47 is a 66 aa protein that was generated by fusing the basic region of MAX to the HLH domain of E47, a member of the bHLH family that is structurally similar to the bHLHZ family but lacks the 25–30 aa leucine zipper contained in bHLHZ proteins.[33,34] E47 heterodimerizes with bHLH family members and serves as the master regulator of their activities.[35,36] MAX shares the same master-regulator role, but only regulates bHLHZ family members; there is no crosstalk between bHLH and bHLHZ proteins. Like MAX, E47 is also capable of homodimerization, and therefore, ME47 was designed to homodimerize and possess the DNA-recognition capability of MAX.

As expected, ME47 bound to the E-box site with high affinity and DNA sequence specificity in both yeast one-hybrid assays (Y1H) and in *in vitro* fluorescence anisotropy titrations.[31] Furthermore, in a modified yeast one-hybrid assay (MY1H),[37] where competitive binding between two transcription factors onto a single DNA target can be assessed, ME47 strongly outcompeted the binding of the MAX bHLHZ homodimer onto the E-box target. This suggested the possibility of the utility of ME47 as an anti-MYC cancer therapeutic, one that acts as a competitive inhibitor against MYC/MAX DNA binding.

The efficacy of using ME47 as an anti-MYC agent was recently demonstrated. Lustig et al. generated an MDA-MB-231 breast cancer cell line that expresses ME47 in response to 4-hydroxytamoxifen (4OHT).[38] Expression of ME47 in these breast cancer cells in cell culture reduced cellular proliferation as compared to the control. In a mouse xenograft using this cell line, ME47 expression in an already established tumor reduced the tumor growth rate by over 60% and increased animal survival. ChIP-seq analysis confirmed binding of ME47 to E-box containing promoters in MDA-MB-231 cells; endogenous MYC occupancy of these promotor regions decreased significantly in the presence of ME47.

Hence, ME47's mechanism of action is believed to be as it was originally designed: to competitively inhibit MYC/MAX binding to the E-box, which is demonstrated by ME47's high-affinity, sequence-specific recognition of the E-box target (K_d 15.3 nM)[31] as well as its E-box-binding profile in the ChIP experiments.[38] The crystal structure demonstrates that ME47 homodimerizes through its E47 HLH.[39] ME47 does not interact with MYC, as shown by yeast two-hybrid (Y2H) assay,[31] and therefore, it is unlikely that the effects observed in the MDA-MB-231 cancer cells resulted from ME47 interfering with the protein-protein interaction of the MYC/MAX heterodimer. These results demonstrate that ME47 is a peptide inhibitor that disrupts interaction of MYC with E-box elements, and ME47 can be used to target transcription factors involved in diseases such as cancer.

2.2.3 | H1 peptide

The H1 peptide (formally known as H1-F8A,S6A) is another MYC inhibitor that was derived from the 14 aa sequence of Helix 1 of the MYC HLH region.[40] Compared to the native MYC Helix 1 sequence, H1 peptide has two functionally nonessential residues substituted with alanines to improve α-helicity. Thus, the H1 peptide was designed to impede dimerization of MYC with MAX, which is necessary for MYC

binding to the E-box site. Indeed, H1 peptide did inhibit a mixture containing MYC and MAX from binding to the E-box site in EMSA.[41] Giorello et al. then demonstrated that a fusion protein comprising H1 and the 16 aa Antennapedia penetratin domain[12,13] entered the MCF-7 breast cancer cell line, causing nearly 10-fold decrease in cancer cell survival while impeding MYC-regulated genes.

The elastin-like polypeptide, ELP, was used to deliver the H1 peptide to tumor cells.[42] ELP is a thermally responsive biopolymer that forms reversible aggregates upon hyperthermia treatment at temperatures slightly above body temperature. This attribute of ELP can be used to deliver ELP-fused peptides into an animal; peptides are systemically administered to the animal, followed by hyperthermia of target tissue. The authors fused the H1 peptide to ELP and the penetratin domain to generate Pen-ELP-H1. In a proof-of-principle study using MCF-7 cell cultures, hyperthermia treatment of cells after addition of Pen-ELP-H1 conjugate to culture media caused two-fold increase in cell death, compared with cells that received Pen-ELP-H1 but no hyperthermia treatment.[42]

Bidwell et al. then optimized the cellular uptake of the peptide by separately fusing the H1 peptide to three different cell penetration domains: Antennapedia penetratin domain, HIV-TAT domain,[43] and Bac cell-penetrating motif.[44] Of the three constructs, the peptide fused to Bac motif (Bac-ELP-H1) showed the most potent activity against MCF-7 cells. In a mouse xenograft model using E0771 breast cancer cells, systemically administered Bac-ELP-H1 was effectively delivered to the tumor upon hyperthermia from infrared illumination that resulted in 70% reduction of tumor volume.[45] Using the same method, Bac-ELP-H1 was tested against a rat model using C6 glioblastoma cells.[46] Bac-ELP-H1 was systemically delivered to tumor-bearing animals, followed by hyperthermia treatment after each delivery, for four consecutive days. This resulted in 80% reduction of tumor volume, allowing 80% of treated animals to survive over 30 days post-tumor implantation, compared to under 40% survival for control animals ($n = 6$–9 animals for each condition). No adverse toxicity or weight loss due to the polypeptide treatment was observed in the animals.

An alternative approach for delivery of the H1 peptide was recently developed by Li et al. by using docetaxel (DTX),[47] a small molecule that slows the rate of cellular mitosis by interfering with microtubule dynamics. DTX was used as pretreatment before delivery of H1 peptide. The cell's nuclear envelope is much more fragile during mitosis; hence, prolonging this phase by use of DTX allows H1 peptide to more easily enter the nucleus. In a mouse xenograft model bearing HeLa cells, administration of DTX followed by the H1 peptide decreased tumor volume by 86%, while greatly increasing the survival rate 40 days post treatment.

3 | SMALL PEPTIDES THAT TARGET TRANSCRIPTION FACTORS

3.1 | HOX inhibitors

The HOX genes (HOX is a contraction for "homeobox") are a group of homologous genes that regulate spatial organization of a developing organism [HOX genes are reviewed in Ref. (48). HOX transcription factors contain the homeodomain, which is the helix-turn-helix motif responsible for recognition of specific DNA target sites. In humans, there are 39 HOX genes organized in four clusters. In addition to their function as regulators of body development, HOX genes also regulate processes such as the maintenance of adult stem cells.[49] Much evidence exists showing that HOX genes are dysregulated in various solid and hematological cancers [HOX and cancer are reviewed in Ref. (50). In the tumor, HOX genes can promote proliferation, block apoptosis, induce angiogenesis, and facilitate drug and radiation resistance. Drug development against these genes is particularly difficult owing to the high degree of functional redundancy among the four HOX gene clusters.

HOX transcription factors can heterodimerize with other homeodomain proteins such as PBX proteins, which are the pre-B-cell leukemia homeobox transcription factor family[51]; these HOX/PBX heterodimers bind to octameric DNA target sequences, with each monomer targeting a DNA half site using its homeodomain. Although HOX proteins can bind to DNA as a monomer, forming a heterodimer with PBX proteins greatly increases the DNA binding affinity and sequence specificity of HOX proteins for their DNA targets; thus, the PBX family can regulate the activities of the HOX family [PBX proteins are reviewed in Ref. (52)].

3.1.1 | HOX hexapeptide

HOX/PBX dimerization is facilitated by a short, 4–6 aa consensus sequence located at the N-terminus of the HOX homeodomain; this HOX hexapeptide reaches over to contact PBX when HOX/PBX is bound to DNA.[53–55] A highly conserved Trp residue in the HOX hexapeptide inserts into the hexapaptide-binding pocket in the PBX homeodomain. The protein-protein interaction between the HOX hexapaptide and PBX homeodomain is required for cooperative DNA binding by HOX/PBX.[53,54] Moreover, Knoepfler et al. showed that it was possible to competitively inhibit HOX/PBX dimerization using a 12 aa synthetic peptide that carries the HOX hexapeptide sequence.

3.1.2 | HXR9

In 2007, Morgan and colleagues generated an 18 aa synthetic peptide, HXR9, by fusing the HOX hexapeptide to nine consecutive Arg residues that facilitate cell penetration of the peptide.[56,57] Treating cell cultures of murine B16 melanoma cells with HXR9 inhibited the HOX/PBX dimer from binding to DNA, as shown by EMSA where the authors examined whether the cell lysate of HXR9-treated cells could cause a mobility shift of a DNA fragment containing the HOX/PBX target site. Furthermore, intravenous administration of HXR9 twice a week for four weeks to mice bearing B16 cells caused over fivefold decrease in tumor growth rate. No adverse side-effects were observed during this treatment. This was the first report showing the efficacy of HXR9 as an anti-HOX therapeutic. Over the last 10 years, HXR9 has been tested against numerous types of cancer cell lines in mouse models. These studies include nonsmall cell lung cancer (A549 cell line),[58] ovarian cancer (SK-OV3),[59,60] breast cancer (MDA-MB-231),[61] melanoma (A375M),[62] meningioma (IOMM-Lee),[63] prostate cancer

(LNCaP),[64] malignant mesothelioma (MSTO-211H),[65] and acute myeloid leukemia (K562).[66] Impressively, HXR9 showed potent antitumor properties against all these tumor types.

Many of these studies also reported the extent of HOX dysregulation in their respective cancer types and analyzed which of the 39 HOX genes were dysregulated in the cells. In many of these analyses, increased levels of c-Fos expression was reported as the cause of apoptotic cell death caused by HXR9. Their analyses illustrated a very high degree of variability for the type of HOX genes dysregulated in each type of cancer cell line. HXR9 is capable of impeding tumor activity despite these differences, owing to the potency of the hexapeptide itself.

3.2 | HIF inhibitors

The Hypoxia Inducible Factor (HIF-1α) is a transcription factor belonging to the bHLH Per-ARNT-Sim (PAS) family of proteins [reviewed in Ref. (67). HIF-1α is activated under hypoxic conditions, and the activated protein forms a heterodimer with its partner protein, aryl hydrocarbon receptor nuclear translocator (ARNT).[68] The HIF-1α/ARNT heterodimer binds to the hypoxia response element (HRE), thereby upregulating expression of hypoxia-induced genes. Genes controlled by HIF-1α include vascular endothelial growth factor (VEGF) and its receptor VEGFR, both of which regulate angiogenesis. In a tumor, dysregulation of HIF-1α greatly impacts oncogenesis, as HIF-1α-regulated genes are involved in angiogenesis, which is vital for tumor growth.

Once the HIF-1α/ARNT heterodimer binds to the HRE target, it initiates gene expression by recruiting a third protein cofactor, p300, to the promoter region of the gene.[69,70] Recruitment of p300 by HIF-1α/ARNT is mediated by the interaction between the C-terminal activation domain (C-TAD) of HIF-1α and the Cys-His-rich CH1 domain of p300. Within the HIF-1α C-TAD domain are two short 6–7 aa α-helices; these helices, designated αA and αB, are essential for the interaction between HIF-1α and p300.

3.2.1 | Modified αA helix

In an attempt to develop an anti-HIF-1α molecule, Arora and coworkers synthesized a short peptide based on the sequence of the C-TAD αA helix.[71] They surmised that the short peptide would interact with the CH1 domain of p300, thereby competitively inhibiting the protein-protein interaction between HIF-1α and p300. This peptide must be properly folded in α-helical structure to be active. However, such short peptides are known to be typically poorly folded.[72] To address this problem, the folding of the αA peptide was enhanced by using the hydrogen bond surrogate (HBS) method.[73] Normally, an α-helix is held together by hydrogen bonds that bridge the backbone C═O group of the *i*th amino acid to the backbone NH group of the $i + 4$th amino acid. Using the HBS concept, Arora and coworkers modified the αA peptide by replacing the hydrogen bond between the first and fourth amino acid with a covalent carbon-carbon linkage that forces the peptide into an α-helical conformation.[71] Using circular dichroism spectroscopy, they confirmed that the modified αA peptide has improved α-helical structure. The modified αA peptide was then tested in HeLa cell culture, and the peptide successfully suppressed VEGF expression. This effect was achieved without causing adverse side effects, which contrasts with other VEGF inhibitors such as the small molecule chetomin.[74]

3.2.2 | HBS 1

Using the same approach, the same group produced another HIF-1α inhibiting peptide, this time using the sequence of the αB helix: HBS 1 possesses the same sequence as the αB helix except for a nonessential Leu substituted with Ala.[75] An attenuated version of HBS 1 was generated as a control by mutating one of the essential leucines in the αB sequence (designated as HBS 2). In a cell-free environment, HBS 1 bound to the p300 CH1 domain with a dissociation constant (K_d) of 690 nM, whereas HBS 2 bound with K_d 3000 nM. The unconstrained version of HBS 1, where its backbone hydrogen bond was not replaced by covalent bonds, bound to the CH1 domain with K_d 6000 nM. The effect of HBS 1 was then evaluated in MDA-MB-231 breast cancer cells that were modified to carry a luciferase reporter gene controlled by the VEGF promoter. Treating these cells with HBS 1 resulted in 25% reduced luciferase signal under hypoxic conditions, as compared to controls. RT-PCR analysis of cells treated with HBS 1 also showed a dose-dependent reduction in the expression of HIF-controlled genes, such as VEGF, SLC2A1, and LOX. Furthermore, the efficacy of HBS 1 was tested in a mouse xenograft model using 786-O RCC human kidney renal cell adenocarcinoma cells. HBS 1 treatment of these mice caused 53% reduction in tumor volume when compared with the controls.

3.3 | BCL6 inhibitors

The BCL6 (B-cell lymphoma) oncoprotein is a transcriptional repressor that regulates the differentiation and maturation of B lymphocytes (B-cells) in the germinal center (GC) reaction. During the GC reaction, naïve B-cells that have been exposed to an exogenous antigen proliferate rapidly. As they proliferate, the gene in these B-cells that encodes the immunoglobulin variable domain undergoes hypermutation. Multiple clones of B-cells are then generated that collectively produce a wide range of specific and nonspecific antibodies against the exogenous antigen. From these B-cell clones, those that produce the most specific antibodies are selected for terminal differentiation [reviews of the GC reaction are found in Refs. (76,77).

One function of BCL6 in the GC reaction is to repress systems that respond to DNA damage.[78] Hypermutation of the immunoglobulin gene inevitably "damages" DNA; damage to DNA would normally cause cell cycle arrest. BCL6 represses genes such as the DNA damage sensor ATR (ataxia telangiectasia and Rad3 related), thereby bypassing DNA repair mechanisms and allowing the hypermutating B-cells to proliferate during the GC reaction.[79] Oncogenesis can result from damage to the cell's DNA. Therefore, during oncogenesis, dysregulated BCL6 allows the proliferating tumor cells to bypass the DNA damage response mechanisms that may otherwise stop tumor growth. Dysregulation of BCL6 is frequently found in large B-cell lymphomas, as well

as in breast cancers; in fact, the oncoprotein was named for the "B-cell lymphoma."[80]

BCL6 is a zinc-finger DNA-binding protein that carries an N-terminal BTB/POZ (Bric-a-brac, Tramtrack, Broad complex/Poxvirus zinc finger) domain.[80,81] BCL6 forms a homodimer through its BTB domain, which forms a groove along the dimerization surface. This groove serves as an interface for protein-protein interactions, including those with corepressors such as SMRT (silencing mediator for retinoid and thyroid hormone receptor) and BCoR (BCL6-interacting corepressor).[82,83] SMRT and BCoR interact with BCL6 through their 17 aa BCL6-binding domains (BBD).[84,85]

3.3.1 | BBD peptide

To target the recruitment of SMRT and BCoR by BCL6, Melnick and coworkers generated the BBD peptide,[86] which is a recombinant protein that comprises the SMRT BBD, plus the HIV-TAT domain for cellular penetration,[43] HA (hemagglutinin) tag for immunodetection, and 6×His tag for purification. They proposed that the SMRT BBD in this peptide would competitively inhibit the protein-protein interaction between BCL6 and its corepressors. In cell cultures of various lymphoma cell lines, the addition of BBD peptide blocked BCL6-mediated gene repression and mediated cell death in BCL6-dependent lymphoma cells.[86] However, the BBD peptide was highly unstable in serum, requiring researchers to add fresh BBD peptide to the cell culture every 4 h for the entire 48 h experimental duration.

The Melnick group made multiple, sequential modifications to the BBD peptide to improve stability.[87] First, they truncated the 21 aa SMRT BBD domain to nine essential residues, and fused this 9-mer to the HIV-TAT domain to facilitate cell penetration. The fusogenic (Fu) HA2 motif from influenza virus hemmagglutinin-2 was also fused to the TAT peptide, because the group realized that these motifs work synergistically to enhance BBD peptide activity. However, the new Fu-TAT-BBD peptide still suffered from lack of stability, which was solved by synthesizing the retro-inverso form of the peptide. Retro-inverso peptides possess the same sequence as their original peptide but are synthesized using D-isoform amino acids.[88] Retroinverso peptides are known to retain the functionality of the original peptide while being more resistant to protease degradation owing to their unnatural isoform.[89] Initially, the retroinverso Fu-TAT-BBD did not show any anti-BCL6 activity because of steric hindrance caused by D-Pro located near the N-terminal end of the peptide. Activity was restored by replacing D-Pro with D-Gly. As a result of these modifications, RI-BPI (retroinverso BCL6 peptide inhibitor) was produced.[87]

RI-BPI retains antitumor activity comparable to the original BBD peptide, while having a vastly improved stability. The addition of RI-BPI in cell cultures of BCL6-dependent DLBCL (diffuse large B-cell lymphoma) cells, such as SU-DHL4 and SU-DHL6, attenuated cellular growth. In this experiment, RI-BPI was applied only *once* to the cell culture at the beginning of the 48 hour incubation period. In a mouse xenograft model using the SU-DHL4 and SU-DHL6 cells, intraperitoneal injection of RI-BPI decreased tumor size by up to 10 times in a dose-dependent manner while also improving the survival rate of animals. Scientists also tested the toxicity of RI-BPI by intraperitoneally injecting the peptide to healthy mice every day for 21 days, or weekly for 1 year.[87] Intriguingly, no signs of toxicity or immunogenicity were observed in both cases. More recently, the effectiveness of RI-BPI was shown against breast cancer cell lines.[90] Application of RI-BPI to cell cultures representing the major breast cancer subtypes (MDA-MB-468, T-47D, SK-BC-3) caused an altered expression of BCL6-controlled genes in all three cell types, while also causing apoptotic cell death in a dose-dependent manner. The effectiveness of RP-BPI against these cell lines are yet to be shown in *in vivo* animal models, however.

4 | SMALL TRANSCRIPTION FACTOR INHIBITING PEPTIDES DERIVED FROM RANDOM SCREENING

All of the peptides described up to this point were developed through rational design that stems from our understanding of the structures and functions of naturally existing proteins. Here, we highlight a recent example where a peptide inhibitor was developed by screening randomized peptide libraries.

4.1 | ERG

ERG (ETS-related gene) is a transcription factor belonging to the ETS (E26 transformation-specific) family of proteins. Members of the ETS family regulate cellular processes such as cell proliferation, differentiation, and apoptosis [ETS and tumorigenesis are reviewed in Ref. (91). ERG dysregulation is frequently caused by a rearrangement event that fuses the ERG coding region to the androgen-regulated promoter region of the TMPRSS2 (transmembrane protease, serine 2) gene, and such dysregulation is frequently associated with prostate cancers. This rearrangement results in constitutive overexpression of a near full-length version of ERG protein from the TMPRSS2 promoter [ETS gene fusions reviewed in Ref. (92).

Wang and coworkers recently used phage display technology to develop an ERG-inhibitory peptide.[93] The screen uses a DNA library cloned into the genome of M13 bacteriophage. This library represents up to 10^9 unique molecules of randomized, 7 aa peptide sequences; individual peptides are expressed on the surface of their respective phage particles. In each round of selection, phage particles were screened for their ability to make specific interactions with the ERG protein via the peptides displayed on their surfaces. After four rounds of selection, 64 phage particles were selected for genome sequencing, yielding a total of 12 unique peptide sequences. Interestingly, a subgroup of the 12 peptides was homologous to the serine-rich (SR) domain of the DLC1 (deleted in liver cancer 1) protein. DLC1 is a tumor suppressor gene that is often deleted in liver, prostate, lung, colorectal, and breast cancers.[94] Two representative peptides selected from this subgroup were designated as ERG inhibitory peptides, EIP1 and EIP2.

In EMSA, EIP1 and EIP2 bound to ERG at 0.6 and 1.8 μM K_d values, respectively.[93] The group then fused the HIV-TAT domain to these peptides to study their activity in cell culture. Both TAT-EIP1 and TAT-EIP2 entered the VCaP prostate cancer cell line, and the peptides caused up to two-fold reduction in the capability of these cells to

invade other tissues. The EIP peptides did not affect the activity of prostate cancer cell lines that do not carry the ERG-mutation (DU145 and PC3), which provides evidence for the specificity of EIP peptides towards their intended target.

To increase stability, retroinverso versions of EIPs were synthesized (RI-EIPs).[93] RI-EIPs showed similar potency in cell culture assays as compared to the L-isoform EIPs. In a mouse xenograft model bearing VCaP cells, daily intraperitoneal injection of RI-EIPs caused a dose-dependent reduction in tumor size, and 10% of tumors completely regressed after 18 days of treatment. Many of the regressed tumors showed no signs of recurrence, both during treatment with RP-EIPs and up to 30 days after the treatment was stopped. RI-EIPs did not have any effect in mouse xenografts carrying the ERG-negative PC3 cell line, which again shows specificity of these peptides. No signs of weight loss or liver toxicity were observed in mice treated with RI-EIPs.

In a cell culture study, treatment of VCaP cells by RI-EIP promoted destabilization and degradation of ERG in those cells. This degradation of ERG was dependent on the presence of a functional proteasome. Thus, the authors hypothesized that RI-EIP inhibits ERG by enhancing its rate of degradation by proteasome-mediated proteolysis.[93]

5 | FUTURE PROSPECTS AND CONCLUSIONS

The field of peptide inhibitors targeting transcription factors has been steadily increasing its productivity and achievements. Many of the studies discussed in this review have shown notable success. Frequently, these peptide therapeutics can effectively control the proliferation of tumor cells, and some even cause complete regression of an already established tumor without signs of recurrence for an extended period of time. At the time of writing, we are unaware of reports that describe testing of these peptides in a clinical setting. Many of these peptides were originally developed in the mid/late 2000s, and since then, their efficacy has been tested *in vitro* and *in vivo*. It appears that the initial, proof-of-principle studies are now concluding for many of these peptides. Indeed, Morgan et al. are planning to enter clinical trials with a variant of HXR9 in 2018.[50] We eagerly anticipate more reports of other peptides advancing to clinical trials, as well.

We find it intriguing, especially from a clinical perspective, that all of the studies discussed above found no side effects when the peptides were tested in mouse models. For example, Omomyc and RI-BPI did not produce observable harmful side effects in the animals, even after a year-long exposure to these peptides. The molecular mechanism for this apparent lack of side effects is not well understood. Regarding the Myc inhibitor Omomyc, Lorenzin et al. proposed that high-affinity E-box sites are necessary for normal physiological processes, but medium- and low-affinity sites are also recognized when elevated concentrations of Myc are present in tumor cells.[95] The interactions between dysregulated Myc and these medium/low-affinity target sites are hypothesized to play a key role in the survival and propagation of a tumor. Thus, even a slight decrease in Myc activity in tumor cells can be highly detrimental, as the low-affinity, potentially disease-causing interactions between Myc and DNA target do not form; hence, researchers have coined the term that cancer cells are "addicted" to Myc. In contrast, the effect of Myc inhibitors is not severe in healthy cells, which do not rely on these low-affinity interactions and also carry intact checkpoints and feedback mechanisms enabling rapid cell-cycle exit and recovery.[96] Although there are not yet many peptides developed against transcription factor targets, this small number of well-studied inhibitory peptides has consistently shown no observable negative side effects in the animal models.

Here, we presented a panel of peptide therapeutics that *directly* inhibit their intended transcription factor targets. Other peptide inhibitors of transcription factors that we did not discuss include the direct and indirect inhibitors of transcription factor NF-κB [reviewed in Ref. (97) and indirect inhibitors of transcription factor Stat3.[98,99] There is yet another class of peptide therapeutics that works by *restoring* the function of a nullified tumor suppressor gene such as p53.[97] Of course, the targets for peptide therapeutics are not limited to transcription factors, but can also include enzymes such as kinases [reviewed in Refs. (97,100). In parallel to the development of peptide therapeutics presented above, other researchers have used the structures of transcription factors and their partner proteins as the basis for generation of peptidomimetics: these molecules resemble the structures of their original peptides and can be used to target disease-causing proteins in similar fashion to peptide therapeutics. For example, peptidomimetic therapeutics have been developed against transcription factors such as HIF-1α [reviewed in Ref. (101) and Stat3 [reviewed in Ref. (102).

Rationally designing peptide therapeutics allows scientists to develop drugs that interact with their targets with high specificity. However, this approach is often challenging. Recently, Edwards et al. attempted to develop a peptide inhibitor against the oligodendrocyte transcription factor OLIG2, which is a bHLH transcription factor that drives the development of glioblastoma [reviewed in Ref. (103). To generate an OLIG2 inhibitor, a library of short peptides based on the sequences of either helix 1 or helix 2 of the OLIG2 HLH were synthesized.[104] These synthetic peptides were stabilized using the hydrocarbon stapling method to enhance the helical structure of peptides by forming covalent bridges between amino acid residues.[105] The resulting series of peptides—stabilized α-helices of OLIG2 (SAH-OLIG2)—were hypothesized to bind to the OLIG2 HLH, thereby disrupting OLIG2 homodimerization and DNA binding. However, these peptides were not capable of specifically interfering with the OLIG2-DNA interaction in EMSA, despite their having increased stability, as shown by circular dichroism spectroscopy.[104] Interestingly, after an attempt to determine why SAH-OLIG2 was not effective, the authors discovered that a region C-terminal to the OLIG2 bHLH was playing an unexpected role in OLIG2 dimerization, suggesting that this C-terminal may be an alternative target for peptide therapeutics.

This paper[104] illustrates a major point in rational design of molecules; rational design is limited by our knowledge of the target, and that knowledge is often built through efforts like these studies. As mentioned earlier, other scientists, such as Wang and colleagues,[93] have approached the field using nonrational methods such as phage display [reviewed in Ref. (106). These nonrational methods have the potential to overcome bottlenecks and difficulties that can stall rational

approaches. We anticipate that the accumulation of knowledge in rational and nonrational peptide therapeutic development, and cross-talk between these approaches, will boost the speed of development of peptides as drugs against disease.

ACKNOWLEDGMENTS

The authors are grateful for funding from NSERC Discovery Grant and the Collaborative Health Research Program (CHRP).

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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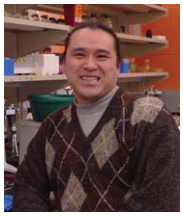

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How to cite this article: Inamoto I, Shin JA. Peptide therapeutics that directly target transcription factors. *Peptide Science*. 2019;111:e24048. https://doi.org/10.1002/pep2.24048