

Received: 2004.11.18
Accepted: 2004.12.17
Published: 2005.02.15

Anti-tumor chemotherapy utilizing peptide-based approaches – apoptotic pathways, kinases, and proteasome as targets

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Source of support: grants from the MHRC, and DFG (Lo 823/3-1). Dr. M. Los is supported by CRC “New Cancer Therapy Development” program.

Summary

The pharmacological sciences are taking advantage of recent discoveries that have defined the molecular pathways governing apoptosis. These signaling cascades are frequently inactivated or distorted by mutations in cancer cells. Peptides derived from critical interaction, phosphorylation, or cleavage sites are the preferred leads (starting points) for the development of new drugs. In this review we summarize recent peptide-based approaches that target MDM2, p53, NF- κ B, ErbB2, MAPK, as well as Smac/DIABLO, IAP BIR domains, and Bcl-2 interaction domains, with a specific focus on the BH3 domain. Separate parts of the review deal with proteasome inhibitors, integrin-derived peptides, and molecules that are being tested for tumor-selective delivery of anticancer drugs (“magic bullet” approach). The proteasome inhibitors and integrin-derived peptides show a variety of effects, targeting not only tumor growth, but also angiogenesis, metastasizing potential, and other cancer cell functions. The last part of this review describes approaches that use specific properties (surface receptors, increased enzymatic activities) of cancer cells in order to target them specifically. These new generations of anticancer drugs provide the foundations for therapies with fewer side effects and higher efficacy.

Key words: **angiostatin • anti-angiogenic • Bortezomib • Velcade • EGFR • Endostatin • HMR1826 • integrins • MDM2 • p53**

Abbreviations: **IAP** – inhibitor of apoptosis protein, **EGFR** – epidermal growth factor receptor, **PCD** – programmed cell death, apoptosis, **UPP** – ubiquitin-proteasome pathway, **BH3** – Bcl-2 homology 3, **BIR** – baculovirus IAP repeat, **MAPK** – mitogen-activated protein kinase, **Smac/DIABLO** – second mitochondrial activator of caspases/direct IAP-binding protein with low pI, **$\Delta\psi$ M** – mitochondrial membrane potential, **TRAIL** – tumor necrosis factor-related apoptosis-inducing ligand, **MM** – multiple myeloma, **PSA** – prostate-specific antigen, **VEGF** – vascular endothelial growth factor, **NLS** – nuclear localization signal, **PTP1B** – protein tyrosine phosphatase 1B, **MDM2** – murine double minute 2, **SH2** – Src homology-2, **CCK** – cholecystokinin, **JNK** – c-Jun N-terminal kinase, **JIP** – JNK interacting protein-1, **KIM** – kinase interacting motif, **B-CLL** – B cell chronic lymphocyte leukemia, **ICAM-1** – intercellular adhesion molecule-1, **c-FLIP** – FLICE inhibitory protein, **MMPs** – matrix-metalloproteinases.

Full-text PDF: http://www.aite-online/pdf/vol_53/no_1/6819.pdf

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INTRODUCTION

Programmed cell death (PCD, apoptosis) is fundamental to the development and existence of virtually every higher organism on earth²⁵. Abnormal regulation of apoptosis, or programmed cell death, has been implicated in a number of human diseases, including cancer^{94, 107}, stroke, myocardial infarction^{49, 68}, viral infections^{22, 36}, and several other diseases⁶⁸. Apoptotic failure can lead to cancer resistance towards chemo- or radiotherapy^{9, 17, 75, 81, 87, 114}. Therefore, molecules and pathways that govern the PCD process have become an attractive target for the development of novel anticancer strategies^{45, 63, 66, 78}. Peptides derived from larger molecules that are

important modulators of apoptosis are frequently becoming leads (primary substances) for the development of anticancer therapeutics (Table 1). Peptide-based (or peptide-derived) anticancer drugs have the potential to selectively target molecules and pathways deregulated in the course of carcinogenesis. Thus this approach offers the potential of non-genotoxic, genotype-specific alternatives or adjuvants to the current regimen of treatments. Such a patient-tailored cancer cell-directed therapeutic approach has the potential to have fewer side effects⁶¹ and to be more effective. Below we discuss targets and approaches to the development of peptide-based cancer therapy. In the first part of the review we will focus on molecules that play a direct role in apoptot-

Table 1. Summary of peptide-based anticancer approaches discussed in this review

Peptide name, sequence, origin	Biological effect
Proteasome inhibitors	
Bortezomib (Velcade™), synthetic peptide	Proteasome Inhibitor, anti-cancer effects through controlling the stability of proteins involved in the regulation of apoptosis, survival, adhesion, angiogenesis, tumor invasion and metastasis ⁹⁰ Anti-inflammatory effect due to inhibition of NF- κ B ⁵ and of adhesion molecules for leukocyte-endothelial cell interaction ⁹⁰
“Magic bullet” peptides and structures	
LTVxPWY, breast cancer cell line SKBR3 binding peptide	Used for the cancer cell-specific delivery of antisense phosphorothioate oligonucleotides directed against <i>erbB2</i> receptor, mRNA (inhibited the target protein expression by 60%) ¹⁰⁴
HMR1826, (N-4- β -glucuronosyl-3-nitrobenzylloxycarbonyl-doxorubicin)	Tumor site-specific delivery of doxorubicin ⁵⁶
β -D-Glc-IPM, (β -D-glucosylisophosphoramidate mustard)	Targeted delivery of cytostatic ifosfamide via glucose transporter SAAT1 ¹⁰⁹
NLS, (-VQRKRQKLMP-NH ₂)	Nuclear transport peptide. It has been successfully used to target NF- κ B, β -galactosidase and several antisense oligonucleotides ⁹⁵
Peptides that target kinase-pathways	
EC-1, WTGWCLNPEESTWGFCTGSF	Binds to the extracellular domain of ErbB2 Acts extracellularly ⁹²
KDI-1, Trx-VFGVSWVVGFWCQMHRRLVC-Trx	Binds to intracellular domain of EGFR, protein based transduction ¹⁶
CIYK,	Bind to p60 c-src, delivery through cell membrane ⁶⁴
CCK-8 analogue, N-acetyl-Asp-Tyr(SO ₃ H)-Nle	Binds to PTP1B, delivery through cell membrane ⁷⁴
Compound 29, 2-[4-[(2S)-2-[(2S)-2-[(tert-Butoxycarbonyl)amino]-3-phenylpropanoyl]amino]-3-oxo-3-(pentylamino)propyl] 2-(1(2H)-tetrazol-5-yl)phenoxy]acetic acid	Binds to PTP1B, delivery through cell membrane ⁷⁴
TI-JIP, KRPTTLNLF	Binds to JNK, delivery through cell membrane ⁷
F56, (WHSDMEWWYLLG), synthetic peptide	Inhibition of VEGF ³
Peptide-based inhibitors of angiogenesis and cell adhesion	
Angiostatin, peptide fragment of plasminogen	Endogenous angiogenic inhibitor ^{39, 89}
Endostatin, peptide fragment of collagen XVIII	Endogenous angiogenic inhibitor ⁸⁸
N-Ac-CHAVC-NH ₂ , synthetic peptide containing HAV motif	Inhibition of type 1 cadherin ³⁴
c(RGDfV), synthetic peptide containing RGD motif	Inhibition of α -v- β 3 integrin ^{4, 20, 84}

Table 1. Continued

Peptide name, sequence, origin	Biological effect
Peptide-based inhibitors of p53-pathway	
53BP2 p53 binding domain: fluorescein-REDEDEIEW	Binds and stabilizes p53 wild-type conformation, activating p53 target genes, and partially restoring p53-dependent apoptosis ^{41, 42, 60}
p53 C-terminal peptide fused to <i>Ant</i> : GSRHSSHLKSKKGQSTSRHKK-KKWKMRNRNQFWVKVQRG*	Partially restores the transcriptional activity of some p53 mutants, and induces p53-dependent apoptosis in tumor cell lines ^{67, 102}
MDM2-binding domain of p53 fused to <i>Ant</i> : PPLSQETFSDLWKLL-KKWKMRNRNQFWVKVQRG	Cytotoxic to tumor cells regardless of their p53-status ⁶⁵
Peptide-based Bcl-2/Bcl-X _L inhibition	
BH3 domain of Bax: KKLSECLKRIGDELDS, BH3 domain of Bak: GQVGRQLAIIGDDINR	Induces apoptotic events in a cell-free assay ²⁶
BH3 domain of Bax: STKKLSECLKRIGDELDSNM, BH3 domain of Bak: GQVGRQLAIIGDDINR	Induces apoptotic events in a mitochondrial assay ⁸⁶
<i>Ant</i> fused to the BH3 domain of Bak: RQIKWFQNRMRMKWKK-MGQVGRQLAIIGDDINRRY	Apoptosis in Hela cells, and resensitization the Bcl-X _L -overexpressing cells to Fas-induced apoptosis ⁵⁴
BH3 domain of Bad (cpm-1285): <i>Decanoic acid</i> NLWAAQRYGRELRRMSDEFEG SFKGL	Apoptosis in HL-60 tumor cells and slowed the growth of human myeloid leukemia in a xenograft model ¹¹³
BH3 domain of Bad fused to 8 Arg: R8NLWAAQRYGRELRRMSDEFVDSFKK, BH3 domain of Bid fused to 8 Arg: R8-EDIIRNIARHLAQVGDSMDR	Bad BH3 and Bid BH3 exhibit synergistic killing of Jurkat leukemic cells ⁷²
BH3 domain of Bak fused to VP-22: MGQVGRQLAIIGDDINRRY-VP22	Targeted, light activated killing of cancer cells ¹²
Hydrocarbon-stapled BH3-domain of Bid: EDIIRNIARHLA*VGD*N _L DRSIW, *non-natural aa attached to the hydrocarbon staple, N _L – norleu	Apoptosis in human leukemia cells and slowed growth of leukemia in a xenograft model ⁹³
Peptide-based IAP inhibition	
Smac/DIABLO peptide: AVPIAQK	Procaspase-3 activation <i>in vitro</i> ²³
Smac/DIABLO peptide: AVPIAQK fused to the TAT protein transduction domain	Sensitized resistant neuroblastoma and melanoma cells, and primary neuroblastoma cells <i>ex vivo</i> to apoptosis induced by TRAIL or doxorubicin, and enhanced the activity of TRAIL in an intracranial malignant glioma xenograft mode ⁴⁴
Smac/DIABLO peptide: AVPI	Sensitizes Jurkat cells to apoptosis induced by TRAIL or epothilone ⁴⁸
Smac/DIABLO peptide fused to 8 R: AVPIAQK-GGGRRRRRRRGCG	Reversed the resistance of H460 cells to cisplatin and taxol, and regressed the growth of H460-derived tumors in mice when co-injected with cisplatin ¹¹⁸

* For the Bcl-2/Bcl-X_L-, p53-, and IAP-pathways, the transport peptides are indicated in italics.

Ant – *Drosophila* Antennapedia homeodomain protein peptide.

ic pathways, including p53, Bcl-2/Bcl-X_L and pro-apoptotic Bcl-2 family members (with special focus on the BH3-domain), inhibitor of apoptosis protein (IAP) family members (focusing on XIAP), and their modulator Smac/DIABLO. In the following section we will discuss approaches that target phosphorylation and ubiquitination-dependent pathways, with the focus on the mitogen-activated protein kinase (MAPK) and epidermal growth factor receptor (EGFR)/ErbB2 pathways as well as on the therapeutic inhibition of the proteasome. Finally, since the

desired anticancer therapy should specifically target cancer cells, we present some peptide-based approaches that have the capacity to (semi)selectively target cancer cells and thus bring us closer towards achieving this goal.

TARGETING THE P53 PATHWAY

p53 is an integral tumor suppressor that regulates genes controlling cell cycle arrest and/or apoptosis. Over half of all human tumors harbor mutations in

the p53 gene⁵⁰, while others are defective in the p53-controlled apoptotic pathway, for example through MDM2 over-expression. Mutant p53 is over-expressed in cancer cells, and the lack of functional p53 can make tumor cells resistant to apoptosis induced by either chemotherapy and/or radiotherapy. Therefore, reactivating the p53 pathway in tumor cells is an obvious therapeutic target¹¹⁴. Three main types of peptide-based therapy are being investigated: stabilizing mutant p53, disrupting the allosteric regulation of p53 by its C-terminal domain, and interrupting the regulatory interaction between p53 and MDM2³⁷.

Structural mutants of p53 could potentially be rescued using peptides that preferentially bind to the native rather than the distorted structure of p53, thereby stabilizing the native structure and allowing p53 to transactivate its target genes. Friedler et al.⁴² screened a series of peptides derived from 53BP2, a protein that binds p53 in its DNA binding site, for their ability to bind and stabilize the p53 core domain *in vitro*. They identified a nine-residue peptide, CDB3, that could restore the specific DNA binding activity of the structural mutant I195T to almost wild-type levels⁴². Fluorescein-labeled CDB3 (Fl-CDB3) was later found to rescue the wild-type conformations of several p53 mutants^{41, 60}, activate p53 target genes, and partially restore p53-dependent apoptosis⁶⁰.

The C-terminal domain functions as a negative allosteric regulator of the p53 tetramer, and interruption of this interaction could activate p53. Selivanova et al.¹⁰² found that a 22-amino-acid peptide (peptide 46) corresponding to the carboxy-terminal amino-acid residues 361–382 of p53 could partially restore the transcriptional activity of some p53 mutants. When peptide 46 was fused with a 17-amino-acid peptide from the *Drosophila* antenapedia homeodomain protein, it was able to induce p53-dependent apoptosis in tumor cell lines with mutant p53 or wild-type p53¹⁰². This peptide was later found to activate p53 by binding to the core domain and displacing the C-terminal domain¹⁰³. The antenapedia-peptide 46 fusion was also found to induce Fas/APO-1-mediated apoptosis in breast cancer cell lines with p53 mutations and over-expressed wild-type p53, but did not induce apoptosis in the absence of p53⁶⁷.

MDM2 binds to p53, targeting it for ubiquitination and degradation⁵³. One potential method to activate p53 would be to interrupt this negative regulation of p53 using peptides. Kanovsky et al.⁶⁵ designed peptides spanning amino acids 12–26 of the MDM2-binding domain of human p53, and found that when fused

to the 17-amino-acid peptide from the antenapedia protein, these peptides were cytotoxic to human cancer cells irrespective of their p53 status, but had no apparent effect on normal human cells⁶⁵. This cytotoxicity was found to be by necrotic cell death, and bio-informatics and biophysical data suggest that this peptide forms a hydrophobic helix-coil-helix structure capable of disrupting cancer cell membranes^{30, 99}. While this cytotoxicity appeared to be specific to cancer cells, it is p53-independent. Therefore, the peptides were not functioning via the p53/MDM2 interaction as expected, emphasizing the need for thorough evaluation of all potentially therapeutic peptides. Kritzer et al.⁶⁹ developed a β -peptide mimic of the MDM2-binding domain of p53. β -peptides differ from normal peptides by one backbone carbon and, because of this, they are more resistant to degradation than α -peptides. This β -peptide mimic of p53 bound MDM2⁶⁹, but as yet no *in vivo* studies have been published.

BCL-2/BCL-X_L AND BH3 PEPTIDES

The Bcl-2 family of proteins are important regulators of apoptosis and include both anti-apoptotic members (such as Bcl-2 and Bcl-X_L) and pro-apoptotic members (such as Bax, Bak, Bad, Bid, and Bik). The family is defined by the presence of at least one of the Bcl-2 family homology (BH) domains (BH1–BH4). These domains regulate the homo- and heterotypic interactions of the family members, interactions that are thought to be critical for the regulation of apoptosis²⁸. High Bcl-2 expression is found in a variety of human cancers and abrogates the normal cell turnover, allowing uncontrolled cell expansion. Furthermore, this Bcl-2-mediated resistance to apoptosis can also decrease the sensitivity of these cells to chemotherapy and radiation therapy. Therefore, Bcl-2 and the related Bcl-X_L protein are targets for novel cancer therapies^{57, 81, 87}. Most of the current research has centered on the BH3 domain of the pro-apoptotic members of the Bcl-2 family because this domain is responsible for heterodimerization with other Bcl-2 family members and the induction of cell death²⁸.

Peptides of the BH3 domains of the pro-apoptotic Bax and Bak proteins were able to induce apoptosis in a cell-free system²⁶, and mitochondrial $\Delta\psi$ M loss, swelling, and cytochrome c release, events important in apoptosis, in isolated mitochondria⁸⁶. Subsequently, Holinger et al.⁵⁴ found that a fusion peptide of the BH3 domain of Bak and the antenapedia protein internalization domain could induce apoptosis in HeLa cells. The over-expression of Bcl-X_L could prevent this apoptotic response, but the Ant-Bak BH3 peptide was still able to re-sensitize the Bcl-X_L-over-

-expressing cells to Fas-induced apoptosis⁵⁴. This Ant-Bak BH3 peptide was also found to induce substantial cell death in erythrocyte cultures¹¹², emphasizing the need for meticulous toxicity testing before any pro-apoptotic peptide is considered for clinical testing. Wang et al.¹¹³ found that a peptide of the pro-apoptotic protein Bad fused to decanoic acid and cell-permeable moiety (cpm)-1285, could enter HL-60 tumor cells, bind Bcl-2, and induce apoptosis, but had minimal effect on normal human peripheral blood lymphocytes. cpm-1285 slowed the growth of human myeloid leukemia in severe combined immunodeficient mice, but caused no gross signs of organ toxicity in normal C57BL/6J mice¹¹³.

Letai et al.⁷² found that there are two subcategories of BH3-only proteins: Bid and Bim induce the oligomerization of the multi-domain pro-apoptotic Bak and Bax proteins, while Bad and Bik bind to Bcl-2. When tagged with polyarginine to facilitate cellular uptake of the peptides, Bad BH3 and Bid BH3 were able to synergistically kill Jurkat cells⁷². Therefore, multiple BH3 peptides could potentially be used to increase the efficacy of this type of treatment.

Brewis et al.¹² have developed a system whereby a peptide can be activated by light following cellular uptake. VP22, a structural protein of the herpes simplex virus, forms complexes called vectosomes with short oligonucleotides. When the BH3 domain of Bak is fused to VP22, the resulting vectosome enters cells and stably resides in the cytoplasm without toxicity until exposure to light. Light activation induces the release of the BH3-VP22 fusion protein from the vectosome, thereby inducing apoptosis. When CT26 adenocarcinoma cells were treated with VP22-BH3 vectosomes and then injected subcutaneously in mice, light exposure of the injection site 24 h later resulted in slowed tumor growth¹².

One of the potential problems with peptide-based therapies is the *in vivo* instability of the peptides. The α -helix of the BH3 domain is critical for its interaction with Bcl-2 and Bcl-X_L⁹³, and a hydrocarbon-stapled BH3 domain of the Bid protein was found to be helical, protease resistant, and cell permeable. It was capable of binding to Bcl-2 and induced apoptosis in human leukemia cells. Furthermore, this modified BH3 domain slowed the growth of human leukemia cells that were injected into severe combined immunodeficient mice, but did not cause any gross toxicity to normal tissue¹¹¹. Lactam-bridged BH3 peptide analogues have also been developed¹¹⁷, as have several Bcl-2 and Bcl-X_L antagonists based on α -helical mimicry^{35, 70, 120}.

IAPs AND Smac/DIABLO

Like Bcl-2 and Bcl-X_L, members of the IAP family are abnormally expressed in some cancers^{58, 94}. The IAP family, which is defined by the presence of one or more baculovirus IAP repeat (BIR) domains, includes X-linked IAP (XIAP), c-IAP1, c-IAP2, and survivin. IAPs selectively inhibit caspase-3, -7, and -9 and can also interact with Smac/DIABLO, an IAP-inhibitory protein that is released from the mitochondria during apoptosis. Smac/DIABLO acts as a pro-apoptotic protein by physically preventing the IAPs from inhibiting caspases⁵⁸. Therefore, peptide inhibitors of the IAPs have been designed from the Smac/DIABLO protein. The four N-terminal residues (Ala-Val-Pro-Ile) of Smac/DIABLO recognize a surface groove on BIR3 of XIAP^{76, 116}, and an N-terminal heptapeptide of Smac/DIABLO can promote procaspase-3 activation *in vitro*²³. Fulda et al.⁴⁴ tagged the seven N-terminal amino acids of Smac/DIABLO with the protein transduction domain of the Tat protein. They found that this fusion Smac peptide sensitized resistant neuroblastoma and melanoma cells and primary neuroblastoma cells *ex vivo* to apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or doxorubicin. Furthermore, this peptide enhanced the anti-tumor activity of TRAIL in an intracranial malignant glioma xenograft model *in vivo*, but had no noticeable toxicity to normal brain tissue⁴⁴. The N-terminal tetrapeptide of Smac/DIABLO has also been found to sensitize Jurkat cells to apoptosis induced by TRAIL or epothilone, an antimicrotubule agent⁴⁸. The N-terminal heptapeptide of Smac has also been conjugated with a poly-arginine tag to create a different cell-permeable IAP inhibitor. This fusion was found to reverse the resistance of H460 cells to cisplatin and taxol and regressed the growth of H460-derived tumors in mice when co-injected with cisplatin¹¹⁸. Interestingly, these Smac-derived peptides have little or no activity without an additional apoptotic signal^{44, 48, 118}. Therefore, targeting downstream proteins in apoptosis, such as the IAPs, may be just as effective as, if not more effective than, targeting upstream events because of this requirement for an additional signal.

TARGETING PHOSPHORYLATION-DEPENDENT SIGNALING PATHWAYS WITH SMALL PEPTIDES

Protein kinases that regulate intracellular signal transduction pathways emerge as another set of molecules that became attractive targets for anticancer therapy. Kinases play an important role in a wide array of cellular processes, including apoptosis, proliferation, and gene transcription. An alteration in

their function can lead to diseases such as cancer, diabetes, and immune disease^{15, 106}. Protein kinases can be divided into those specific to tyrosine phosphorylation (tyrosine kinases) and those specific to serine or threonine phosphorylation (serine/threonine kinases). Drugs such as ST I-S71/Gleevec target the ATP binding site of kinases²¹. However ATP is a common substrate for many enzymes; these compounds could inadvertently inhibit other signaling pathways. An alternative approach is to inhibit the interaction of kinases with their substrates by using small-molecule analogues of these substrates.

Receptor tyrosine kinases include members of the epidermal growth factor receptor family such as EGF receptor (EGFR) and ErbB2. These receptors are over-expressed in several tumors and correlate to poor prognosis^{16, 92}. Their elevated expression levels and lack of a physiological role in adults make them prime targets in cancer treatment. Extra-cellular targeting of ErbB2 has been a success story in the treatment of breast cancer, as is the case of the humanized 4D5 antibody trastuzumab (also known as Herceptin)⁹². Pero et al.⁹² utilized a similar approach; they identified peptide EC-1, which was able to target the extra-cellular domain of ErbB2 and inhibit the phosphorylation of its intracellular functional domain. EC-1 was also observed to inhibit the proliferation of ErbB2-over-expressing cells⁹². Intracellular targeting of kinases is more difficult since proteins generally have poor cell membrane permeability. Buerger et al.¹⁶ utilized a novel approach for introducing inhibitors into the cell. They generated a peptide specific to the kinase domain of EGFR and fused the peptide sequence to the bacterial protein thioredoxin. These types of fusion proteins are called aptamers. The fusion locked into position the 3-dimensional conformation of the short peptide, allowing for more specificity to EGFR. The aptamers were produced in bacteria and were introduced into the cell by adding a protein transduction domain to its sequence. Protein transduction is not fully understood, but it is a process in which extracellular proteins are unfolded, carried across the membrane, and refolded intracellularly without loss of activity¹⁶. One drawback of using such large proteins is that they might eventually trigger an immune response in the patient, leading to degradation of the protein.

Kamath et al.⁶⁴ generated peptide inhibitors specific to the enzyme-substrate interaction site of the non-receptor tyrosine kinase p60 c-src. p60 c-src is involved in proliferation and mitosis, and its deregulation leads to neoplasticity. This group was able to generate cysteine-containing hexa- and heptamers that were strong inhibitors of p60 c-src kinase activi-

ty. These peptides had the sequences Cys-Ile-Tyr-Lys-Tyr-Tyr-Phe and Cys-Ile-Tyr-Lys-Tyr-Tyr, respectively. Their rationale for the addition of cysteine groups was that herbimycin A, a strong inhibitor of p60, contained many sulfhydryl groups that are important for its function. To improve cell permeability, they generated the shorter, more hydrophobic tetramer Cys-Ile-Tyr-Lys, which retained relatively high inhibitory properties. This compound can serve as a basis for future drug development⁶⁴.

Another approach to inhibiting tyrosine kinases is by targeting Src homology-2 (SH2) domains. These domains recognize and bind to proteins possessing a phosphorylated tyrosine^{73, 100}. Peptidomimetic compounds are designed to imitate sequences of target proteins containing a phosphorylated tyrosine¹⁰⁰. One problem with these molecules is that phosphotyrosine groups are very susceptible to chemical and enzymatic degradation¹⁰⁰. Burke et al.¹⁸ designed a number of phosphotyrosine analogues that have high stability. This led to the discovery of *c*-phosphomethylphenylalanine, a phospho-tyrosine mimetic that is not only phosphatase resistant, but also has very similar biological properties to phospho-tyrosine⁷³.

Tyrosine phosphorylation is a dynamic process that is controlled by the opposite actions of kinases and phosphatases. Phosphatases remove the phosphate group from phosphorylated proteins¹⁹. Some groups have focused on targeting and inhibiting phosphatases in order to prolong the signal generated by active protein kinases^{10, 74}. In type II diabetes, many tissues are desensitized to the effects of insulin. Tyrosine phosphatase 1B (PTP1B) dephosphorylates active insulin receptors; thus the inhibition of PTP1B will prolong the signal generated at the insulin receptor. Analogues of the protein cholecystokinin (CCK), such as N-acetyl-Asp-Tyr(SO₃H)-Nle, are potent inhibitors of PTP1B^{10, 74}. This phosphatase recognizes a moiety containing a phospho-tyrosine residue, which was initially replaced with an ortho-sulfate group, which is more resistant to degradation. Subsequent research determined that the phosphate biostere *o*-malonate and a novel biostere 2-carboxymethoxy benzoic acid yielded more potent inhibitors. However, addition of these biosteres resulted in decreased permeability into the cell^{10, 74}. Liljebris et al.⁷⁴ reported that replacing the carboxylic group with an ortho-tetrazole analogue made compounds more cell permeable, at the same time maintaining high inhibitory potency¹⁰.

MAPKs are serine threonine kinases that act through signaling transduction cascades relaying information

from the cytosol to the nucleus^{7, 8}. c-Jun N-terminal kinase (JNK) is a MAPK that is involved in processes such as apoptosis, survival, and tumor development. Efforts have focused on utilizing the domains of interactive partners in order to inhibit JNK. One of these interactive partners is the scaffold protein JIP. It has been observed that over-expression of JIP protein itself can inhibit JNK. TI JIP is a potent inhibitory peptide that resembles the kinase interaction motif (KIM) of JIP^{7, 8}. Bonny et al. overcame cell permeability problems by engineering a biological peptide inhibitor of JNK by linking the 20-amino-acid inhibitory domain of JIP-1 to a 10-amino-acid HIV-TA cell-permeable sequence. This peptide was imported into pancreatic B TC-3 cells and blocked JNK-mediated activation of c-Jun¹¹.

PROTEASOME INHIBITORS: THEIR POTENTIAL IN THE TREATMENT OF CANCER AND INFLAMMATION

Proteasome inhibitors are a relatively new class of drug that target the proteasome, a multicatalytic proteinase complex, thereby disrupting intracellular protein degradation. It is estimated that 80% of cellular protein turnover occurs through the ubiquitin-proteasome pathway (UPP)⁹⁰. Proteins are designated for this process by the enzymatic addition of a polyubiquitin chain, which is recognized by the proteasome. Normal homeostasis is compromised in the cell by preventing degradation of inactive or dysfunctional proteins and interfering in other essential cell processes such as protein maturation, immune function, mitosis, angiogenesis, and apoptosis. Interestingly, it appears that malignant cells are more sensitive to the deleterious effects of proteasome inhibitors than are their non-transformed counterparts. However, Masdehors et al.⁸³ found that in B cell chronic lymphocyte leukemia (B-CLL), only specific proteasomal inhibitors, such as lactacystin, discriminated between cancerous and normal lymphocytes, while the less specific MG132 (a tripeptide aldehyde that can also inhibit calpains) did not.

Mechanisms of antineoplastic activity

Of the various processes affected by proteasome inhibitors, 4 have been described as the major contributors to their anti-cancer effect: adhesion, angiogenesis, apoptosis, and tumor invasion and metastasis⁹⁰.

Adhesion. Proteasome inhibitors target molecules involved in cellular adhesion, such as P-selectin, lymphocyte function-associated antigen-1, endothelial cell adhesion molecule, and intercellular adhesion molecule (ICAM)-1. In multiple myeloma, the pro-

teasome inhibitor bortezomib (Velcade, formerly PS-341) reduced myeloma cell adherence to bone marrow stroma, which in turn decreased stromal production of myeloma growth and survival factor IL-6. The effect of chemosensitization to standard myeloma therapies is also seen, as adhesion contributes to chemotherapy resistance. Inhibiting expression of P-selectin, ICAM-1, and E-selectin expression on vascular endothelial cells mediates an anti-inflammatory response by decreasing leukocyte-endothelial cell interactions, which is beneficial in the clinical scenarios of vascular occlusion and ischemic events. There is also potential for use in situations of inflammation present with *Streptococcal* cell wall-induced polyarthritis.

Through population-based studies, a link between chronic inflammation and susceptibility to cancer has been observed⁵. It is proposed that in precancerous cells, NF- κ B enhances cell survival and pre-malignant potential by augmenting the expression of pro-inflammatory and survival genes while inhibiting death-promoting machinery. Proteasome inhibitors prevent translocation of NF- κ B to the nucleus by stabilizing the inhibitory protein I κ B α , suggesting therapeutic potential. However, it should also be noted that in the skin, inhibiting NF- κ B actually promotes one type of cancer due to a reduction in terminal differentiation in keratinocytes.

Angiogenesis. Proteasome inhibitors exert a direct anti-angiogenic effect on vascular endothelial cells by blocking the production of molecules such as plasminogen activator and vascular endothelial cell growth factor (VEGF) receptors. Suppressing tumor cell production of the pro-angiogenic cytokines, VEGF, and growth-related oncogene- α also contributes to inhibition of angiogenesis. Resultant anti-tumor effects have been noted in human and murine squamous cell carcinoma *in vivo* as well as in model systems of multiple myeloma and breast, pancreatic, prostate, and colon carcinomas. Other anti-tumor approaches that target tumor-stimulated angiogenesis are described in the next section of this review.

Apoptosis. The proteasome controls the stability of proteins that regulate cell cycle progression and apoptosis in normal and malignant cells. Some examples include cyclins, NF- κ B, caspases, Bcl-2¹, p44/42 MAPK, JNK, p53, c-FLIP, and TRAIL death receptors 4 and 5⁹⁰. Upregulation of DR 4 and 5 and down-regulation of c-FLIP contribute to the activation of caspase-8, which is an upstream initiator of both the intrinsic and extrinsic pathways of apoptosis^{90, 63}. NF- κ B is a ubiquitous transcription factor that is constitutively activated in a variety of solid tumor cancers

(breast, prostate, colorectal, and ovarian) as well as in hematologic malignancies such as Hodgkin's disease, certain leukemias, and multiple myeloma. Inhibition of NF- κ B decreases the transcription of genes involved in proliferation, survival, invasion, metastasis, and angiogenesis. The p44/42 MAPK signal transduction cascade is involved in a variety of cellular processes, including growth and survival in response to mitogenic stimuli, angiogenesis, tumorigenesis, invasion, and transformation in certain malignancies. Inhibiting p53 degradation can induce apoptosis through Bax to promote mitochondrial release of cytochrome c, or cause cell cycle arrest through p21. Yang et al.¹¹⁹ have shown that treatment of non-small cell lung cancer cells with bortezomib activated the JNK/c-jun/AP-1 signaling pathway and upregulated p21^{waf1} and p53, inducing growth arrest and apoptosis.

Tumor invasion and metastasis. Studies of the Lewis lung carcinoma model have shown the potential of proteasome inhibitors to decrease lung metastases to 22–35% of vehicle-treated controls, with a lower percentage of large vascularized metastases. Invasion was also blocked in a modified Boyden chamber assay using prostate carcinoma cell lines. These observations are likely due to the previously discussed effects on apoptosis and angiogenesis⁹⁰.

Clinical application of proteasome inhibitors

Clinical trials with the single agent bortezomib. Bortezomib is the first proteasome inhibitor to reach clinical trials. It has shown both *in vivo* and *in vitro* activity in a variety of malignancies, and its efficacy does not seem to be influenced by the presence of known drug resistance factors. Six phase I clinical trials, involving approximately 200 patients to date, with both solid tumors and hematological malignancies have been completed or are underway². Toxicities included grade 3 diarrhea, sensory neuropathy, fatigue, headache, anemia, neutropenia, arthralgias, fever, and moderate hyponatremia and hypokalemia. Results ranged from stabilization of patients with melanoma, nasopharyngeal carcinoma, and renal cell carcinoma, to a 50% reduction in measurable disease burden and symptoms in a bronchoalveolar lung cancer patient, and complete and durable remission in a patient with advanced, heavily pre-treated multiple myeloma (MM)⁹⁰. A positive effect on serum prostate-specific antigen (PSA) in advanced androgen-independent prostate cancer patients was observed⁹¹, and *in vitro* studies have shown decreased PSA in addition to growth arrest and apoptosis in androgen-dependent human prostate cancer LNCaP cells⁵⁹.

Results in phase II studies in MM led to fast-track approval of bortezomib by the US Food and Drug Administration in May 2003, and promising data have been obtained for the treatment of non-Hodgkin's lymphoma⁴⁶. However, grade 3 and 4 toxicities and poor response in advanced renal cell carcinoma in one study have led to a recommendation to discontinue studies in this disease, although results from other studies are still pending⁹⁰. Phase III trials with refractory MM patients comparing bortezomib treatment with an increased dosage of dexamethasone were terminated early due to a significantly longer time to disease progression with bortezomib⁹⁰.

Potential for combination therapy. Proteasome inhibitors can contribute to the sensitization of cancer cells to the activity of radiation and chemotherapy. Clinical trials involving combinations of bortezomib with thalidomide or pegylated liposomal doxorubicin in MM patients have shown promising preliminary results with 57 and 73% response rates, respectively, in patients known to be resistant to these agents alone. In both cases this was a higher response rate than would be expected for single agent bortezomib therapy⁹⁰. Chauhan et al.²⁴ studied cases of refractory MM patients who were initially sensitive to bortezomib but ultimately developed resistance to the drug and found that combining bortezomib with triterpenoid CDDO-Im triggered a synergistic apoptotic response. Synergistic results have also been noted in non-small cell lung cancer cells when bortezomib was combined with the histone deacetylase inhibitor sodium butyrate²⁹, and phase I and II trials with a combination of bortezomib/gemcitabine/carboplatin are underway⁷¹. Combining bortezomib with the cyclin-dependent kinase inhibitor flavopiridol led to a marked increase in mitochondrial injury, caspase activation, and synergistic induction of apoptosis in myelomonocytic leukemia cells²⁷.

Immediate perspectives of proteasome inhibitor-based therapy. Due to the central role of the UPP in regulating cellular processes and success in clinical trials, the proteasome inhibitors have already entered the clinic as novel anti-cancer therapeutics. Bortezomib has been approved for treatment of refractory multiple myeloma and is undergoing further investigation to determine its potential for use in both solid tumor and hematologic malignancies. It has also shown potential in combination therapy due to its ability to sensitize cancer cells to the effects of standard therapies. Due to the key role of the UPP in the management of the protein pool in the cell and to the numerous other clinical trials that are already in progress (see above), the approval of other drugs that are based on the same principle and target not only can-

cer, but possibly also inflammatory processes, becomes very probable in the near future.

TARGETING TUMOR ANGIOGENESIS WITH PEPTIDES

Angiogenesis, or neo-vascularization, refers to the process in which new blood vessels are formed from the surrounding pre-existing blood vessels. This process is fundamental to embryogenesis, but plays only a minor role in healthy adults, excluding the female reproductive tract. Abhorrent angiogenesis is involved in various disease states and contributes to joint destruction, blindness, and tumor lethality, to name a few. Angiogenesis is regulated by a balance of pro- and anti-angiogenic molecules. It is a critical step in tumor progression, since a tumor cannot surpass a critical size (2–3 mm³) or metastasize to another organ without developing its own vasculature^{20, 38}. In fact, most tumors in humans persist *in situ* for months to years until a subset of cells within the tumor are switched to an angiogenic phenotype through perturbation in the balance of local pro- and anti-angiogenic factors^{38, 40}. In 1971 Folkman postulated that tumor growth and metastasis were dependent on angiogenesis and that therefore blocking angiogenesis could be a strategy to arrest tumor growth. Tumor-induced angiogenesis is a multi-step process and therefore offers several different targets for therapeutic interventions⁵². Anti-angiogenic drugs stop the formation of new vessels but do not attack healthy vessels, and in theory should do no harm to blood vessels serving normal tissues³⁹. Instead, anti-angiogenic therapy aims to shrink tumors and prevent them from growing. In 1992 the first anti-angiogenic cancer drug, TNP-470 (a synthetic analogue of the substance fumagillin), entered clinical trials³⁹. To date, many different anti-angiogenic drugs have been developed and are in various stages of clinical testing.

VEGF is a well-characterized positive regulator of angiogenesis and is the only mitogen that specifically acts on endothelial cells through interactions with its cell surface ligand, Flk-1. VEGF is major target for anti-angiogenic therapy because its overexpression has been associated with vascularity, poor prognosis, and aggressive disease in most malignancies³². The peptide F56 (WHSDMEWWYLLG), which specifically binds to VEGF, is able to nearly abolish VEGF binding to its receptor, Flk-1, *in vitro*. *In vivo*, this peptide is able to inhibit tumor growth and metastases³. Another drug, bevacizumab, is a monoclonal antibody against VEGF in clinical trials as an anti-angiogenic treatment for metastatic colon cancer³³.

Angiostatin and endostatin are two endogenous inhibitors of angiogenesis. Angiostatin, which is

a fragment of the larger protein plasminogen, is among the most potent of known angiogenesis inhibitors³⁹. It instructs endothelium to become refractory to angiogenic stimuli⁸⁹. Systemic administration of human angiostatin potently inhibits the growth of primary carcinomas in mice without detectable toxicity or resistance⁸⁹. Endostatin is an angiogenic inhibitor composed of the C-terminal fragment of collagen XVIII. It specifically inhibits endothelial proliferation and potently inhibits angiogenesis and tumor growth⁸⁸.

Matrix-metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases that are capable of degrading essentially all components of the extracellular matrix. Degradation of the extracellular matrix is required for neo-vascularization and is therefore a target of anti-angiogenic therapy. Two of the most prominent and well studied inhibitors of MMPs are the compounds batimastat 2 and marimastat 3, which are both broad-spectrum MMP inhibitors with low IC₅₀ values. However, neither compound proved to be effective in clinical trials due to a lack of oral availability and toxicity at high doses, respectively⁸⁴.

Cadherins are a family of transmembrane glycoproteins mediating calcium-dependent homophilic cell-cell interactions. They are regulators of cell motility and proliferation. Classical cadherins (type I) contain a highly conserved sequence at their homophilic binding site consisting of His-Ala-Val (HAV). It has been shown that peptides containing the HAV motif are able to inhibit cadherin function. The cyclic peptide *N*-Ac-CHAVC-NH₂ can perturb cadherin-mediated endothelial cell interactions, resulting in progressive apoptotic death³⁴. E-cadherin is localized at epithelial junctional complexes and participates in the organization and maintenance of epithelia. The ability of carcinomas to invade and metastasize largely depends on the degree of epithelial differentiation within the tumor. Poorly differentiated tumors are associated with poorer prognosis compared with well-differentiated tumors. Selective loss of E-cadherin expression is associated with tumor invasion and metastasis^{43, 101}. E-cadherin is thought to act as an invasion suppressor *in vivo*^{43, 82, 110} and therefore may be a useful marker of tumor invasion potential⁶².

Integrins are a class of cell surface glycoproteins which act as receptors to mediate cell-cell and cell-matrix interactions. All integrins are heterodimers comprised of an α and a β subunit. Individual integrins are cell-type specific and have their own binding specificity and signaling properties. The key integrin involved in angiogenesis, and therefore an anti-angiogenic drug target, is the $\alpha_v\beta_3$ -integrin (vit-

ronectin receptor). This integrin mediates the adhesion of endothelial cells to the extracellular matrix and allows for endothelial cell migration through interactions with the tripeptide motif RGD (Arg-Gly-Asp)⁸⁴. *In vivo* screening of phage display libraries has yielded peptides with the amino acid RGD that preferentially recognize tumor vessels in mice. These peptides can then be used to target therapeutic agents to tumors²⁰. Several cyclic and linear peptides have been developed to antagonize the $\alpha_v\beta_3$ -integrin. For example, a cyclopentapeptide containing the RGD sequence, c(RGDfV), was developed that specifically inhibited the $\alpha_v\beta_3$ -integrin with an IC₅₀ value of 50 nM⁴. This peptide was shown to be effective in inhibiting tumor neo-vascularization in tumors implanted in chick embryos¹⁴. A derivative of c(RGDfV) is currently in the phase I/II stages of clinical trials for the treatment of glioblastoma multiforme (NCI clinical trials). Antagonists of α_v -integrin have also been shown to reduce capillary proliferation in hypoxia-induced retinal neo-vascularization without obvious side effects⁵¹. A recent study has demonstrated that addition of the RGD tripeptide to endostatin can further increase the ability of endostatin to inhibit tumor growth¹²¹.

Peptides blocking angiogenesis are emerging as potential treatments for both cancer and non-neoplastic diseases characterized by aberrant angiogenesis. Preliminary results suggest that anti-angiogenic therapy needs to be administered for a longer time course (several months to a year or more) compared with conventional chemotherapy; however, resistance to inhibitors has not been observed during long-term studies in animals³⁸. Early trials also indicate a synergistic effect of administering a combination of anti-angiogenic and cytotoxic therapy. Folkman³⁹ postulates that this synergy between cytotoxic and anti-angiogenic therapy may be due to the fact that there are two types of cells in tumors (endothelial and tumor cells) and that they respond differently to therapy. Future research in the field of anti-angiogenic therapy using small peptides holds the promise of better treatment for many diseases.

PEPTIDE-BASED STRATEGIES FOR TUMOR TARGETING: THE “MAGIC BULLET” APPROACH

The “magic bullet” approach towards cancer treatment envisioned in the 19th century by Paul Erlich implies developing effective and efficient strategies of chemotherapy⁸⁵. The heterogeneity and susceptibility to develop drug resistance of many cancers have made curative chemotherapy a major challenge. Current studies on successful intracellular delivery and tumor targeting of small selective peptides and

peptide-conjugated molecules have shown promise, although much remains to be done to elucidate their effectiveness. The selectivity of tumor targeting peptides is drawn from exploiting the inherent characteristics of tumors while minimizing cytotoxicity of normal and surrounding tissues. In the advent of gene arrays and peptide phage libraries, high-throughput identification of over-expressed genes and peptides in malignancies has potential applications in developing selective clinical therapies⁹⁸. Recently, Shadidi and Sioud¹⁰⁵ listed numerous target-specific peptides ascertained using phage display libraries.

One targeting approach relies on phenotypic differences between normal and cancer cells using site-directed delivery of prodrugs that are enzymatically activated or released *in situ*. Cytotoxic metabolites of pharmacological agents such as ifosfamid or anthracyclines are released on target tissues when conjugated to peptides that are substrates of cleavage enzymes such as glycosidases and glucuronidases, found abundant in tumor microenvironments^{56, 85, 109}. Other prodrugs can also be linked to peptides or glucose polymers that have specific affinities to receptors with the premise that malignant cells over-express these cell surface receptors and glucose transporters^{85, 109}. Such systems allow tissue-specific delivery and release of drug moiety with minimal effect on normal tissues. Other studies have also used antibody-directed therapy as targeting vehicles for cargoes such as radionuclides and toxins, but further clinical testing and approval are needed to see significant success in this approach⁵⁵. Tumor targeting using peptide carriers is not limited to an array of prodrug agents, but also includes shuttling of oligonucleotides. Anti-neoplastic agents have since expanded to delivering antisense oligonucleotides targeted towards aberrant tumor suppressor genes or oncogenes. These agents are conjugated to peptides such as poly-L-lysine and protamine or packaged in carrier molecules such as cationic-coated liposomes^{13, 77, 115}. Targeted annihilation of tumor cells is also achieved by introducing immunogenic or suicide genes. One example is gene delivery of non-mammalian cytosine deaminase that promotes cell death by interfering with the transcriptional and translational mechanisms of cancerous cells³¹. Delivery of inert viral vectors containing genes encoding thymidine kinases that phosphorylate nucleoside analogues that then disrupt cancer cell proliferation has also been investigated³¹. More detailed reports on peptide delivery of oligonucleotides are provided in recent reviews^{77, 80}. Essentially, naked oligos are prone to nuclease degradation, and peptide delivery of oligonucleotides helps promote increased stability, efficient cellular uptake, and tissue-targeted specificity.

Ideally, a peptide should be both specific to target tissues and itself have anti-neoplastic properties, such as in the monoclonal antibody herceptin targeting erbB2 receptor over-expressed in tamoxifen-resistant breast tumors¹⁰⁸. Unfortunately, there are a limited number of peptides that have such features reported to date. Other delivery approaches make use of peptides as shuttles, as they are easily synthesized, allow tissue penetration with specificity, and can be attached to prodrugs as well as short oligonucleotides. Several documented model peptides bind cell surface receptors over-expressed in tumors. Clinical applications of somatostatin receptors, neurotensin receptors, bombesin and hormone binding receptors, to name a few, have been explored^{47, 56}. The use of covalently conjugated fusogenic peptides that contain nuclear localization signals (NLS) have also shown promise in transporting oligonucleotide cargoes to the nucleus. Most are derived from viruses, such as Tat or SV40 T antigen⁹⁷, or other proteins, such as. antennapedia homeodomain. Recently, the NLS of transcription factors such as Oct-6, TCF1- β , or NF- κ B in the delivery of covalent fusion peptides into MCF7 cells have also been tested^{77, 95}. Furthermore, intracellular transport of peptides and oligos can be enhanced through delivery via colloidal carriers such as liposomes that protect against degradation and can vary tissue specificity by the addition of charged or polymer coatings^{77, 80}.

The current consensus is that the development of targeted therapies has proven difficult due to the heterogeneous presentation of various cancers. Peptide-based strategies for tumor targeting, though encouraging, do present drawbacks. Natural peptides have low bioavailability and short half-life in the mammalian circulation system, while synthetic peptides have potential cytotoxicities¹⁰⁵. Systematic testing in *in vivo* as well as *in vitro* settings must be done rigorously to verify peptide applications in the clinic.

EPILOGUE

Targeting protein-protein interactions within the apoptotic pathways with peptide-based cancer therapies could provide novel, non-genotoxic alternatives or adjuvants to current treatment protocols.

Optimally, the peptide should be preferentially toxic to cancer cells and/or work through a defined genetic pathway that is hyperactive in malignant cells. The studies reviewed above have shown that p53-targeted peptides, BH3 peptides, Smac peptides, peptidomimetics that target the kinase pathways, and proteasome inhibitors all have the potential to enhance the apoptotic response of cancer cells to chemotherapy. The effect, at least in some cases, is selective towards transformed cells or towards the blood vessels within the targeted tumor. Therefore these molecules/approaches either alone or, more likely, as a component of combined therapeutic strategies are bringing us closer to the ultimate goal of anticancer therapy that both selectively targets cancer cells and is effective enough to eradicate the malignancy. While new therapeutics that both selectively target cancer cells and induce PCD is the ultimate goal, the last part of our review indicates that a combination of an unspecific or tumor-semi-specific “killer molecule” and a “vehicle” that selectively delivers it to the malignant cell may be an equally appealing tactic to reach the ultimate goal of cancer cell eradication.

The third approach, not discussed in our review but equally plausible, is to induce tumor-specific immune response. In such a case, the patient's own immunocompetent cells, such as natural killers, lymphokine-activated killers, and neutrophils, would become the effectors of the immune system directed towards eradication of the malignancy. Unfortunately, the immune system, at least in patients with advanced cancer, tolerates the malignant cells despite the expression of several mutated proteins. Conventional chemotherapy kills cancer by an apoptotic process that (contrary to necrosis) assures the “clean” removal of dying cells. Necrotic cell death is, however, capable of inducing an immune response against cellular components that differ from the healthy cells in the body. Since nowadays we have the molecular tools to modulate, even switch between, both forms of cell death⁷⁹ as well as the means to detect it *in vivo*^{6, 96}, the development of immunotherapy protocols either as monotherapy or as an adjuvant treatment is likely to occur in the near future.

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