Increasing the range of drug targets

Interacting peptides provide leads for the development of oncoprotein inhibitors

Bernd Groner,* Axel Weber and Laura Mack

Georg Speyer Haus, Institute for Biomedical Research, Frankfurt am Main, Germany

Keywords: oncoprotein addiction, peptide ligands, interference with protein interactions, tumor cell inhibition

Two limiting aspects are mainly responsible for the sluggish development of new cancer drugs. They concern the chemical properties of potential drug molecules and the structural prerequisites for drug targets. The chemical properties which are being considered desirable for potential drugs are rather restrictive and mainly dictated by the rules of oral availability. Drug target structures are mostly defined as molecules which comprise binding pockets for low molecular weight compounds. These low molecular weight compounds then serve as leads for the derivation of analogs which recognize the same site and which can function as competitive or irreversibly binding inhibitors. The extension of the range of drug targets and the design of suitable lead compounds will be one of the most challenging tasks for drug developers in the future. Such auxiliary drug target structures can be found in the complex networks of interacting proteins which constitute the intracellular signal transduction cascades. The transient assembly of high molecular weight complexes, based on the specific interactions of particular domains, and usually regulated by secondary modifications, propagates extracellular signals through the cytoplasm and into the nucleus. Aberrations in the formation of protein complexes, or in the regulation of their disassembly, often trigger pathological conditions. The interference with interactions of proteins or the interactions of proteins with DNA offer new opportunities for drug discovery and development. Protein complexes which are indispensable for the growth and survival of cancer cells, proteins to which these cells are “addicted,” appear most suited for such an approach. Stat3 and Survivin have been used as model proteins. Specific peptide ligands able to recognize and suppress the functions of crucial protein complexes which do not exhibit these features are usually considered as non-druggable.9,10 The development of technologies which would allow the exploitation of the large repertoire of molecules with a crucial functional domain of a target protein, (2) the induction of a desired cellular phenotype upon intracellular interaction of the peptide ligand with its target structure and (3) the replacement of the peptide ligand with a functionally equivalent low molecular weight, drug-like compound and its optimization through medicinal chemistry.

Principles and Limitations of Drug Development

Insights into the biochemical and functional properties of signaling components, the detection of genetic alterations of genes encoding these proteins and associations of such deregulated protein products with disease states, provide a large number of new therapeutic targets and ample opportunities for drug design. Despite these discoveries, the number of new drugs reaching the market remains disappointingly low, the development periods are long and the costs are rising. Innovative strategies are needed to exploit new drug targets and to interfere with their functions through new classes of molecules. Important prerequisites are already in place to evaluate novel targets and the effects of specific inhibitors. RNA interference is a method which quickly and reliably can yield information on the consequences of the suppression of a particular protein function in cultured cells. Advanced genetically engineered mouse models are available which allow rather precise predictions of particular drug effects in these animals. These techniques also help to evaluate the benefits of combinations of targeted drugs.11,12 A large number of molecules has been identified which are functionally involved in the etiology and progression of cancer.5,6 These molecules are potential drug targets. The most promising among them are proteins which are indispensable for the growth and survival of cancer cells but whose inactivation can be tolerated, at least for a short time period, by normal cells.13 The majority of these “addiction” molecules, however, does not fit the description of conventional drug targets. Such targets are usually enzymes and receptors in which hydrophobic amino acids form binding pockets allowing the access of low molecular weight compounds and the formation of stable complexes.9 Proteins which do not exhibit these features are usually considered as non-druggable.14 The development of technologies which would allow the exploitation of the large repertoire of molecules with
crucial functional roles in pathological processes, but suboptimal characteristics of conventional drug targets, would be of great value.

Biological macromolecules might become useful tools for this purpose. Proteins and peptides, with suitable secondary structures, can be selected as specific ligands for nearly any target protein domain. Particular protein interaction domains are required to allow for protein complex formation. If a peptide exhibits similar binding properties as one of the physiological interaction partners, it can act as a competitive inhibitor of protein interactions. Two types of peptides can be taken into consideration for this purpose: peptides which are directly derived from one of the two interacting proteins and peptides which are selected from a random peptide library. The competitive inhibitors are able to mask an interaction domain and make the domain inaccessible for the natural interaction partner which in turn is required for proper function and finally for the implementation of a particular cellular phenotype. In a next step, these peptide ligand interactions with crucial domains of target proteins can be further exploited in high throughput screening systems to find low molecular weight compounds which act as functionally equivalent analogs.

Desirable Target Structures for the Development of Cancer Drugs: Stat3 and Survivin

We have chosen two target molecules as models which seem particularly attractive for the development of non-conventional cancer drugs: Stat3 and Survivin. Stat3 is a transcription factor with a phosphorylation dependent mode of activation and a well-defined domain structure. Growth factor receptor and cytokine receptor associated tyrosine kinases phosphorylate Stat3 and allow the formation of dimers which translocate to the nucleus, bind specific DNA response elements and modulate transcription. The extent and the duration of Stat3 activation is tightly controlled and tyrosine phosphatases, protein inhibitors of activated Stat and suppressor of cytokine signaling proteins cause a rapid downregulation in normal cells. In contrast, Stat3 is persistently tyrosine phosphorylated in most tumor cells, either through the enhancement of activating signals or the loss of inactivating ones. In tumor cells, sphingosine-1-phosphate receptor-1 upregulates Jak2 activity and leads to enhanced Stat3 phosphorylation. Stat3 in turn regulates sphingosine-1-phosphate receptor-1 and IL-6 and leads to a positive feed-forward loop in epithelial tumor cells. Stat3 activation contributes to many hallmarks of cancer and regulates differentiation, proliferation, apoptosis, the sensitivity toward cytotoxic agents, angiogenesis, recruitment of immune cells and metastasis. The choice of Stat3 as a promising target molecule is further supported by experiments in which Stat3 expression has been suppressed by RNA interference and growth suppression and induction of apoptosis have been observed.

Survivin is a regulatory protein of 142 amino acids and a member of the family of inhibitors of apoptosis proteins (IAPs). It assumes versatile functional roles and participates in the control of apoptosis, cell division, cell migration and metastasis. Several splice variants have been described. Survivin also has an intriguing expression pattern. It is expressed and required for normal fetal development, but not present in most adult tissues. The survivin gene is positively regulated by transcription factors like β-catenin/TCF-β, HIF1α, Sp4 and Stat3 and negatively by the tumor suppressor genes p53, Rb and PTEN. The survivin protein is post-translationally modified by the poly-lie kinase 1, the aurora B kinase and p34cdc2/cyclin B and is also regulated through ubiquitination. It is also involved in the control of cell division. Its expression level is modulated during the G2/M phase of the cell cycle, and survivin is closely associated with centrosomes and the mitotic spindle microtubules. In contrast to its low expression in normal adult tissues, survivin is readily detected in a wide variety of cancers. Survivin probably exerts its anti-apoptotic function upstream of the effector caspases by inhibiting caspase 9. The preferential expression of survivin in cancer cells and its functional role in tumor cell survival and proliferation make it a potential target for tumor therapy. Downregulation of survivin expression in tumor cells with RNAi increased apoptosis and slowed cell growth.

The peptide aptamer technology has been extensively used to identify peptides with binding specificities for particular functional domains of target proteins. Peptide aptamers are short peptides, usually 12 to 20 amino acids in length, which can be selected from random, high-complexity peptide libraries in yeast two-hybrid screens. Our experiments have shown that a variant of the human thioredoxin (hTrx), devoid of cysteine residues, provides a favorable scaffold for the display of such target interacting peptides. Appropriately appended scaffolds allow the presentation of the peptide in a constrained conformation, the production as recombinant proteins and the cellular delivery of peptides which inhibit survivin interaction partners. We found a novel interacting protein, a variant of the human thioredoxin (hTrx), devoid of cysteine residues, provides a favorable scaffold for the display of such target interacting peptides. Appropriately appended scaffolds allow the presentation of the peptide in a constrained conformation, the production as recombinant proteins and the cellular delivery of specific peptide aptamers. The recombinant Stat3 specific peptide aptamer (rS3-PA) was analyzed in detail and the consequences of its cellular uptake into cultured cells and the molecular mode of Stat3 inhibition were investigated. rS3-PA efficiently enters cells, causes the reduction of Stat3 phosphorylation and enhances the proteasomal degradation of P-Stat3. This results in Stat3 target gene inhibition and impaired tumor cell proliferation, migration and survival. Intravenous administration of rS3-PA into mice showed that the systemic application of rS3-PA inhibits Stat3 activation in vivo and slightly retarded the growth kinetics of transplanted glioma cells.

Inhibition of Stat3 and Survivin through Peptide Ligands

An alternative strategy was used to derive a survivin inhibiting molecule. A cDNA library was screened in a yeast two-hybrid setting for survivin-interacting proteins. Several survivin interaction partners have been described, for example, the pro-apoptotic protein Smac or the h oralidin component of the chromosomal passenger complex. We found a novel interacting protein, a domain of the ferritin heavy chain 1 (FTH1). Ferritin is a widely expressed protein, it can sequester free intracellular iron and
embodies ferroxidase activity. It can convert Fe²⁺ to the less reactive and less toxic Fe³⁺. This function involves the coordinated activity of ferritin heavy chain (FTH1) and ferritin light chain subunits, a 24 subunit complex, co-expressed in a large variety of cells.

We integrated the survivin-interacting domain of FTH1 into a recombinant protein, rSip. This protein was also provided with a protein transduction domain and tags which allow for the purification of the bacterially expressed construct and immunofluorescence detection. We investigated the potential of the peptide-based survivin inhibitor rSip in breast cancer (SKBR-3, MDA-MB-468) and glioblastoma (T98G) cells. These cells express high levels of survivin and cause tumor cell death. Non-transformed NIH/3T3 and MCF10A cells remain unaffected. These effects are very similar to the ones induced by downregulation of survivin expression with specific siRNA. rSip provides a lead structure for the development of drugs targeting the tumor cell “addiction protein” survivin.

Discovery of Low Molecular Weight Inhibitors of Peptide Protein Interactions

Peptide sequences can be identified in yeast two hybrid screens which very specifically interact with essential domains of target proteins. If the inhibition of the target protein triggers a particular phenotype in cells, these peptide sequences can be validated as functional inhibitors upon expression in cells or upon transduction of recombinant proteins into cells. These cell culture experiments, however, cannot easily be extrapolated to the in vivo situation. The short half life of the recombinant proteins in the circulation of mice, less than 10 min, precludes that an inhibition of, e.g., Stat3 can be achieved, which lasts long enough, to exert growth inhibitory and apoptotic effects. Even repeated intravenous administration only caused a partial suppression of Stat3 phosphorylation and a modest retardation of tumor cell growth but was insufficient for tumor cell death induction. For these reasons it seems reasonable to replace the peptides by low molecular weight compounds which exert the same functional effects but exhibit better pharmacokinetic properties and bioavailability. The peptides can serve as tools in screening procedures designed to identify such functional analogs. The screen can be based on the disruption of the interaction between, e.g., Stat3 and the peptide apotamer, or the binding between survivin and the FTH fragment. The conditions have to allow for high throughput approaches and the identification of suitable molecules from a low molecular weight compound library of high complexity.

The Alpha Screen, amplified luminescent proximity homogeneity assay, is based on fluorescence resonance and used to detect the interaction of two molecules. For this purpose each one of the interaction partners is being conjugated with dextran or hydrogel beads which contain photosensitive molecules. The binding of the partner molecules to each other brings the donor and acceptor beads in close proximity. The donor bead is then excited with a laser light of 680 nm, the energy is transferred from the donor bead to the acceptor beads via a reactive singlet oxygen and a fluorescent signal of 520 to 620 nm can be measured. The addition of a low molecular weight compound able to disrupt the interaction between the two partners, leads to the extinction of the emitted signal. The systems can be tailored for high throughput and complex compound libraries can be screened. A further improvement of this technology is based on the use of Europium integrated into the donor beads. HYDRO-FRET, homogenous time resolved fluorescence energy transfer, uses a delay in the timing between excitation and emission to minimize false positive results based on spurious interactions. These measurements can be further verified by BiFC, bimolecular fluorescence complementation. This technique is based on the complex formation of two fragments of a fluorescent dye, e.g., yellow fluorescent protein, YFP. The two interacting partners, linked to the YFP fragments, bring the fragments into immediate vicinity of each other which leads to the reconstitution of its fluorescent potential. This can be measured through excitation at 513 nm and emission at 527 nm. Inhibitors of the interaction would interfere with the fluorescence. Intercellulose with BiFC is a method which allows the detection of the disruption of an intracellular protein interaction, a situation most favorable and suited for drug discovery and development.

From Interacting Peptide Ligands to Drug-Like Molecules

The strategy which could lead to a significant extension of useful drug targets and the discovery of suitable lead compounds is outlined in Figure 1. Protein-protein interactions or protein-DNA interactions are based on macromolecular surfaces which usually do not comprise low molecular weight compound binding pockets and therefore do not immediately suggest potential leads. Such interactions are schematically shown in the upper part of the figure. A particular protein domain interacts with a corresponding domain of a binding partner (left) or a specific DNA sequence can be recognized by a defined interaction domain of a DNA binding protein (right). The choice of the target protein is based on biological information about their functions. Crucial nodes in signaling pathways can be identified by RNA
Identification of inhibitors for non-conventional drug targets based on protein interaction surfaces

Non-conventional drug targets
- Protein-protein interaction surfaces
- Protein-DNA interaction surfaces

Identification of specifically interacting proteins or peptides
Source: protein domain of interest, cDNA libraries, synthetic libraries (peptide aptamers)
Screening procedures: e.g. yeast-two-hybrid, FRET or phage-display assay
Interacting properties: e.g. immunoprecipitation, pull-down assays (qualitative) and Biacore measurement (quantitative)

Delivery of peptide sequences into cultured cells
- Integration into gene transfer vectors, e.g. lentiviral infection
- Expression as recombinant protein and protein transduction
- Phenotypic consequences of target protein inhibition (target gene expression, proliferation, apoptosis)

Derivation of low molecular weight compounds with corresponding functional properties
Tools: interacting protein (peptide domain) and peptide, low molecular weight compound library
Screening procedures: 1. Fluorescence resonance energy transfer - Alpha Screen, FRET, HTR-FRET (in vitro) 2. Bimolecular fluorescence complementation – BiFC (in vivo)

Optimisation of compound properties
- Medicinal chemistry
- Bioavailability
- Pharmacokinetics- and dynamics
- Toxicity

DRUG
interference experiments and the phenotypic consequences of the suppression of such proteins can be evaluated in cultured cells. In the case of targets useful in cancer therapy, “addiction molecules,” signaling components which are indispensable for the survival of tumor cells, but not necessarily for the survival of normal cells, seem most appropriate. The transcription factor Stat3 fulfills these prerequisites and can serve as an example in this scheme. Stat3 activation is absolutely required for the growth and survival of many cancer cells and its domain structure is well defined. It comprises clearly delimited dimerization and DNA binding domains. These functional domains of Stat3 can be used as “baits” in interaction assays, yeast two hybrid screens or phage display assays, and peptides sequences can be identified which specifically recognize, e.g., the dimerization domain or the DNA binding domain. Random peptide libraries, peptide aptamors, or cDNA libraries can serve as sources of sequences from which a specific peptide ligand can be selected. The specificity and affinity of the interaction between the selected ligands and the target protein domains of interest can be assessed in co-immunoprecipitation experiments, pull down assays or Biacore measurements.

The binding specificity of the selected peptide ligands in vitro does not yet allow conclusions about their functional properties and their inhibitory potential. The desired outcome, i.e., the competition for binding, the replacement of the physiological binding partner, protein or DNA, and thus the functional inhibition of the target protein has to be tested in cellular assays. For this purpose the peptide sequence can be integrated into a gene expression vector and delivered into cancer cells. The constitutive or conditional expression of such a vector should then suppress the function of Stat3 by preventing its dimerization or DNA binding potential. The suppression of Stat3-dependent gene transcription, growth arrest and induction of apoptosis in certain cancer cells.

Peptides and small proteins usually exhibit suboptimal pharmacokinetic properties in vivo and it might be advantageous to replace them with low molecular weight, drug like compounds, of similar functional properties. A second screening step might allow the substitution of the peptides ligands. These screening procedures are based on the disruption of the interaction between the functional target protein domain and the peptide ligand identified in the first step. Here, the functional domain of the target protein and the peptide ligand are coupled to fluorescent resonance carrier beads introduced into screening assays based on FRET and HTR-FRET. Low molecular weight compound libraries are then used in high throughput settings and tested for their ability to disrupt the fluorescence resonance signal, lower part of the figure. Compounds which interact with the target protein domain and function as competitive inhibitors of peptide ligand binding, can serve as leads for optimization to become drug candidates.

References
1. Orlando A, Pardalina A. Personalised therapies in the cancer “omics” era. Nat Clin Pract Oncol 2008; 5:553-9; PMID:18528979; http://dx.doi.org/10.1038/ncponc.2007.23.
2. Landes Bioscience. Do not distribute
25. Li GH, Wu H, Lo SQ, Ji H, Wang DL. Knockdown of STAT5 expression by siRNA suppresses growth and induces apoptosis and differentiation in glioblastoma stem cells. J Oncol 2010; 3:193-10; PMID:20744360.

26. Ryan BM, O’Donovan N, Dally MJ. Survivin: a new target for anti-cancer therapy. Cancer Ther 2009; 7(suppl 1):S47-52; PMID:19559786; http://dx.doi.org/10.4169/cancerther.2009.05-035.

27. Lladser A, Saito T, Kowalewski B, Quat AM. In survivin the potential Achilles’ heel of cancer? Adv Cancer Res 2011; 111:1-37; PMID:21784529; http://dx.doi.org/10.1016/B978-0-12-807524-4.00001-5.

28. Dole T, Behrens E, Wall JR, Prusa J, Akhurst DC. Microtubule survivin inhibits apoptosis and promotes tumorigenesis. J Cell Sci 2006; 119:1377-87; PMID:16749953.

29. Self RJ, Lopez-Chavez A, Carina D, Jurek HE, Morris JG. Inhibiting tumor cell lines by targeting the inhibitor of apoptosis protein survivin. Mol Cancer 2011; 10:25; PMID:21740246; http://dx.doi.org/10.1186/1476-4268-10-25.

30. Akhurst DC. New insights in the survivin networks. Oncogene 2008; 27:6775-84; PMID:18931605; http://dx.doi.org/10.1038/onc.2008.303.

31. Akhurst DC. Survivin and IAP proteins in cell death mechanisms. Biochim J 2010; 430:199-205; PMID:20744371; http://dx.doi.org/10.1042/BCJ20100014.

32. Islam A, Kyoguwa H, Tahaka N, Kaminishi T, Takahashi H, Inoue E, et al. High expression of Survivin mapped to 17q25 is significantly associated with poor prognostic factors and promotes cell survival in human immunodeficiency. Oncogenesis 2009; 19:1617-23; PMID:19689906; http://dx.doi.org/10.1038/ons.2010.358.

33. Wang T, Wu J, Qiao X, Ding Y, Yu L, Liu B. Gambogic acid, a potent inhibitor of survivin, reverses docetaxel resistance in gastric cancer cells. Cancer Lett 2008; 262:114-22; PMID:18060786; http://dx.doi.org/10.1016/j.canlet.2008.12.004.

34. Xing J, Jia CR, Wang Y, Guo J, Cai Y. Effect of shRNA targeting survivin on ovarian cancer. J Cancer Res Clin Oncol 2012; 138:1221-8; PMID:22520061.

35. Bruegger C, Koech F, Grosner J. Peptide aptamer- or antibody- mediated cancer therapy. Curr Opin High Throughput Screen 2008; 11:135-42; PMID:18536207; http://dx.doi.org/10.1016/j.sher.2007.07.002.

36. Nagal-Wolfstein E, Baeuerle C, Witting J, Bats K, Hopp-Seychel F, Grosner J. The interaction of specific peptide aptamers with the DNA binding domain and the dimerization domain of the transcription factor Stat3 inhibits transactivation and induces apoptosis in human cancer cells. Mol Cancer Res 2004; 2:170-82; PMID:15437906.

37. Borok E, Hymowitz SG, Coughan AG. The mitotic regulator Survivin binds as a monomer to its functional interactor Borealin. J Biol Chem 2007; 282:35018-23; PMID:17881355; http://dx.doi.org/10.1074/jbc.M706233200.

38. Hentze MJ, Thal DR. Cellular regulation and molecular interactions of the ferritins. Cell Mol Life Sci 2006; 63:591-600; PMID:16465450; http://dx.doi.org/10.1007/s00018-005-5285-y.

39. Kunz C, Borghouts C, Buerger C, Groner B. Peptide aptamers with binding specificity for the intracellular domain of the ErbB2 receptor interfere with AKT signaling and sensitize breast cancer cells to Taxol. Mol Cancer Res 2006; 4:983-98; PMID:17189388; http://dx.doi.org/10.1158/1541-7786.MCR-06-0046.

40. Mackie DI, Roman DL. Development of a novel high-throughput screen and identification of small-molecule inhibitors of the Gα-RGS17 protein-protein interaction using AlphaScreen. J Biomol Screen 2011; 16:869-77; PMID:21680864; http://dx.doi.org/10.1177/1087057111410427.

41. Kerppola TK. Bimolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells. Annu Rev Biophys 2008; 37:605-87; PMID:18573981; http://dx.doi.org/10.1146/annurev.biophys.37.050806.125942.