REVIEW ARTICLE

Targeting Survivin in Cancer: Novel Drug Development Approaches

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Abstract Survivin is a well-established target in experimental cancer therapy. The molecule is over-expressed in most human tumors, but hardly detectable in normal tissues. Multiple functions in different subcellular compartments have been assigned. It participates in the control of cell division, apoptosis, the cellular stress response, and also in the regulation of cell migration and metastasis. Survivin expression has been recognized as a biomarker: high expression indicates an unfavorable prognosis and resistance to chemotherapeutic agents and radiation treatment. Survivin is an unconventional drug target and several indirect approaches have been exploited to affect its function and the phenotype of survivin-expressing cells. Interference with the expression of the survivin gene, the utilization of its messenger RNA, the intracellular localization, the interaction with binding partners, the stability of the survivin protein, and the induction of survivin-specific immune responses have been taken into consideration. A direct strategy to inhibit survivin has been based on the identification of a specifically interacting peptide. This peptide can recognize survivin intracellularly and cause the degradation of the ligand–survivin complex. Technology is being developed that might allow the derivation of small molecular-weight, drug-like compounds that are functionally equivalent to the peptide ligand.

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1 Introduction

1.1 Progress in Tumor Therapy and Properties of Desirable Drug Targets

Progress in prevention and therapy has led to remarkable decreases in mortality and death rates due to cancer. Between 1990 and 2008, the death rates declined by 15.1 % in women and 22.9 % in men [1, 2]. Preventive measures, extensive screening programs for breast and colon cancer, and the development of new and effective drugs contributed to these reductions. Studies of the genetic basis of cancer, insights into the regulation of signaling pathways and their biochemical components, understanding the communication between cancer cells and normal cells, and the elucidation of the mechanisms of metastasis are areas in which basic research has made remarkable progress. This knowledge led to the identification and exploitation of new and promising drug targets. Molecularly targeted therapies, aimed at individual signaling components activated in cancer cells, have improved the success of treatment [3, 4].

Following the pioneering example set by the inhibition of the Abelson kinase in chronic myelogenous leukemia patients [5], most of these targeted drugs have been directed against protein kinases that are aberrantly activated in particular cancer cells. The combined treatment of metastatic melanoma patients with selective B-Raf and mitogen-activated protein extracellular kinase (MEK) inhibitors significantly improved their progression-free survival [6]. An inhibitor of ALK (the anaplastic lymphoma kinase) caused durable responses in patients with ALK-positive non-small-cell lung cancer [7]. Patients with myeloproliferative disease benefited from treatment with Janus kinase 2 (JAK2) inhibitors [8] and B-cell

hematologic malignancies responded favorably to the inhibition of the phosphatidylinositide 3-kinase (PI3K) p110δ isoform [9].

Not only kinase inhibitors, but also monoclonal antibodies have become most effective in cancer treatment. Antibodies directed against the epidermal growth factor (EGF) receptor family initially showed the benefit of this class of molecules for cancer therapy [10]. In the meantime, combinations of monoclonal antibodies and conventional chemotherapeutic agents have improved treatment [11] and additional, valuable targets and drug combinations are being exploited. For example, an antibody directed against the cytotoxic lymphocyte antigen CTLA4 caused the reactivation T-cell cytolytic activity against melanoma cells [12] and the combination of antibodies and kinase inhibitors is being used in breast cancer patients [13]. The inhibitor of the hedgehog signaling pathway, vismodegib, resulted in very favorable responses in patients with locally advanced and metastatic basal cell carcinoma [14].

Despite the impressive success achieved with the drugs described above, a large number of promising drug targets have not yet been exploited and offer opportunities for future progress. This holds for many of the oncogenes and tumor suppressors that have been known for many years and which became the foundations of molecular oncology. Mutated versions of, for example, tumor-suppressor protein p53 (TP53), K-Ras, and N-Ras are recognized drivers of transformation in a large number of cancer entities, but targeted drugs exploiting these genetic aberrations are not yet available. The same is true for transcription factors that drive the transformed phenotype, e.g., MYC, N-MYC, signal transducer and activator of transcription 3 (Stat3) and Stat5, and a variety of docking molecules and adapters which play crucial roles in the assembly of high molecular-weight protein complexes. The development of drugs targeting such oncogenic proteins, proteins with an intracellular location and no enzymatic activity, will depend on the development of methods that allow the exploitation of defined protein–protein interactions (PPIs). The disruption of such interactions poses conceptional and technological challenges. A number of encouraging examples, however, show that such an approach is feasible and useful [15–19]. It involves steps that are "non-conventional" in current drug development protocols [20], but it is clearly worthwhile pursuing. Many more, functionally essential oncoproteins could thus become useful drug targets.

1.2 Extending the Range of Useful Drug Targets

The development of drugs usually depends upon suitable drug targets, which meet defined structural prerequisites. Drug target structures are preferably molecules that contain binding pockets for known low molecular-weight compounds. These compounds can serve as leads and structural analogs can be derived that recognize the same site. They can function as competitive or irreversibly binding inhibitors for naturally occurring ligands. The number of drug targets which fulfill these requirements are limited and additional drug target structures have to be explored and exploited. The functions of intracellular signal transduction components usually rely on specific interactions of particular protein domains, and are often regulated by secondary modifications. They propagate extracellular signals through the cytoplasm and into the nucleus and participate in transcription, translation, and organelle function. Aberrations in the regulation of the formation or disassembly of protein complexes are reasons for pathologic conditions. Conversely, the targeted interference with interactions of proteins or the interactions of proteins with DNA, important, for example, for the survival and proliferation of tumor cells, can be used for drug discovery and development.

Cancer cells harbor multiple genomic and epigenetic abnormalities. They also show the persistent activation of particular signaling pathways which are only transiently active in normal cells. These enhanced and prolonged signaling events can result in the dependence on particular activated signaling components for survival and growth [21]. The downregulation or functional inhibition of such molecules often results in the arrest of proliferation or the induction of apoptosis of tumor cells. A number of proteins and protein complexes which are indispensable for the growth and survival of cancer cells, components to which tumor cells are addicted, have been identified. RNA interference (RNAi) experiments can be carried out to downregulate such molecules and verify that they are indispensable in the context of particular cultured tumor cells [22]. They appear most suited as drug targets, and survivin belongs to this class of molecules. Since survivin fits the description of a non-conventional drug target, indirect strategies have been employed to interfere with its function. They are based on the inhibition of transcription of the survivin gene, interference with survivin messenger RNA (mRNA) utilization, the folding or the stability of the survivin protein, its secondary modifications and intracellular localization [23–25]. However, additional efforts are underway to develop drugs that directly interact with survivin and inhibit its functions, i.e., survivin-specific ligands with inhibitory potential [26]. These strategies have been summarized in Fig. 1.

2 Literature Search

A literature search of the biomedical literature was performed via PubMed up to June 2013 using the following

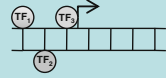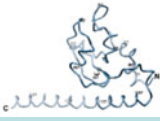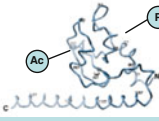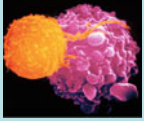## Interference with the biogenesis and function of survivin

Synthesis and processing of survivin	Promoter and survivin gene	mRNA	Protein stability	Protein folding	Secondary modifications	Immune functions
Mode of action	Inhibitors of transcription factors	Stability and utilization of mRNA	Signaling pathways regulation, protein degradation	Chaperone HSP90 interactions	Phosphorylation and acetylation, subcellular localisation and complex formation	Induction of CD8 + cytotoxic killer cells
Molecules and compounds	• ILF3/p54(nrb) • Sp1 • Stat3 • NF-κB • Sox2 • TF acetylation	• antisense oligonucleotides • siRNA (RNA interference) • miRNA (miRNA control)	inhibitors of: • EGFR pathway • Raf-1 • MEK/ERK • rSip (peptide ligand)	• geldanamycin • shepherdin	inhibitors of: •Aurora-B • PLK-1 • CBP • HDAC 6	• peptide vaccination • DC cell loading
references	Nakahara et al. 2007 Castro-Gamero et al. 2013 Zhang et al. 2013 Guha et al. 2010 Feng et al. 2013 Chowdhury et al. 2011	Carrasco et al. 2011 Church and Talbot 2012 Kelly et al. 2011 Hendruschk et al. 2011 Cao et al. 2013 Weiss et al. 2012	Smith et al. 2000 Tecleab and Septi 2013 Weiss et al. 2012	Reikvam et al. 2009 Gyurkocza et al. 2006 Siegelin et al. 2010	Chu et al. 2011 Wang et al. 2010 Riolo et al. 2012 Holloway and Altura 2012	Becker et al. 2012 Widenmeyer et al. 2012

Fig. 1 Interference with the biogenesis and the function of survivin [26]. The majority of the strategies employed to inhibit survivin are based on insights into the regulation of its synthesis and processing. Transcription factors have been identified which interact with and regulate the transcription of the survivin gene. Specific inhibitors for these transcription factors, e.g., ILF3/p54(nrb), Sp1, Stat3, and NF-κB, have been used to downregulate survivin mRNA expression. Upon transcription, the stability and utilization of survivin mRNA can be modulated by molecules able to form double-stranded nucleic acids. Antisense oligonucleotides, siRNA, and miRNA can cause the degradation of survivin mRNA or impede its translation into protein. Several signal transduction pathways are able to affect the stability of the survivin protein through the regulation of E3 ligases and proteasomal degradation. Especially the EGFR pathway and its downstream effectors can be used to manipulate survivin levels. The only molecule whose inhibitory action is based on its direct interaction with survivin is rSip. This molecule is comprised of a survivin interaction domain derived from the ferritin heavy chain. Intracellular binding to survivin causes its degradation and functional inhibition [26]. The proper folding of survivin is dependent upon the chaperone protein HSP90. Its function can be inhibited by geldanamycin and shepherdin, and the subcellular localization of survivin can be manipulated through the interference with kinases and protein acetylases. Finally, survivin-expressing cells can be eliminated through the induction of specific cytotoxic killer cells. *CBP* cyclic adenosine monophosphate response element-binding protein, *DC* dendritic cell, *EGFR* epidermal growth factor receptor, *ERK* extracellular signal-regulated protein kinase, *HDAC* histone deacetylase, *HSP* heat shock protein, *ILF* interleukin enhancer-binding factor, *MEK* mitogen-activated protein extracellular kinase, *miRNA* microRNA, *mRNA* messenger RNA, *NF-κB* nuclear factor kappa B, *PLK-1* polo-like kinase-1, *rSip* recombinant survivin interacting protein, *siRNA* small interfering RNA, *Sp1* specificity protein 1, *Stat* signal transducer and activator of transcription

terms: survivin, survivin cancer, survivin review, survivin apoptosis, survivin inhibitor.

3 Properties of Survivin and the Rationale for Targeting Survivin in Cancer Therapy

Survivin is a regulatory protein of 142 amino acids and a member of the family of inhibitors of apoptosis proteins (IAPs) [27]. A transcript and four-splice variants have been detected. The protein is distinguished by a single baculovirus IAP repeat (BIR) domain, but lacks the RING (really interesting new gene)-finger domain and the caspase-associated recruiting domain (CARD) present in other members of the IAP family. It assumes versatile functional roles and participates in the control of cell division, apoptosis, the cellular stress response, and also cell migration and metastasis [28–30].

Survivin has an intriguing expression pattern. It is expressed and required for normal fetal development, but it is not present in most adult tissues, exceptions being vascular endothelial cells and hematopoietic cells [31]. The

survivin gene is positively regulated by transcription factors such as β-catenin/TCF-Lef, hypoxia-inducible factor 1-α (HIF1α), specificity protein 1 (Sp1) and Stat3, and negatively by the tumor suppressor genes *p53*, *Rb*, and *PTEN* (phosphatase and tensin homolog) [32]. The survivin protein is post-translationally modified by the polo-like kinase-1 (PLK-1), the aurora B kinase and p34cdc2/cyclin B, and is also regulated through ubiquitination [33].

Survivin is present in different subcellular compartments, in the cytosol, the mitochondria and the nucleus, and exerts distinct cellular functions in those compartments. The nuclear survivin is active in the regulation of mitosis and contributes as a chromosomal passenger complex protein to the proper alignment of chromosomes, chromatin-associated spindle formation and kinetochore microtubule attachment [34]. In its acetylated form, it also participates in the formation of transcription complexes [35]. As a regulator of apoptosis in the cytoplasm, survivin interacts with and stabilizes phosphorylated X-linked inhibitor of apoptosis protein (XIAP), and inhibits caspase-3 and caspase-9. In the mitochondria it sequesters pro-apoptotic Smac (second mitochondria-derived activator of caspases) and prevents its release into the cytoplasm [36].

When normal cells and tumor cells were compared, the selective expression pattern of survivin attracted the attention of tumor biologists. No or very little survivin was found in normal tissues, but strong expression was associated with nearly all cancer tissues [37]. Survivin expression in tumor cells could possibly be induced by Wnt and mammalian target of rapamycin (mTOR) signaling [38, 39]. The loss of tumor suppressor proteins and the activation of oncoproteins probably cooperate in the upregulation of survivin in cancer cells. The over-expression of survivin promoted the survival of aneuploid cells [40], blocked caspase-dependent and -independent cell death, increased proliferation, and increased angiogenesis [24]. It also correlated with therapy resistance and unfavorable prognosis in many tumor entities [41, 42].

A number of studies have addressed the consequences of interference with survivin expression or function in tumor cells. The introduction of a dominant negative variant of survivin into prostate and cervical cancer cells caused the formation of multipolar mitotic spindles, failure of cytokinesis, the formation of multinucleated cells, and resulted in reduced proliferation and the induction of apoptosis [43]. The essential contribution of survivin for the survival of cancer cells was corroborated by RNAi experiments and confirmed its potentially valuable role as a drug target. Downregulation of survivin efficiently inhibited tumor cell growth [26] and increased treatment-induced apoptosis of cancer cells [44, 45].

4 Pharmacologic and Genetic Approaches to Interfere with Survivin Function

After it became clear that survivin is a promising therapeutic target in cancer, efforts have been made to develop strategies and compounds able to functionally interfere with this molecule. These efforts, however, have been hampered by the structural properties of survivin, which initially put survivin into the category "non-druggable". Nevertheless, a number of approaches for survivin inhibition have been employed which are based on indirect mechanisms, e.g., interference with the expression of the survivin gene, the utilization of its mRNA, the intracellular localization, the interaction with binding partners, the stability of the survivin protein, and the induction of survivin-specific immune responses (Fig. 1).

4.1 Interference with Survivin Gene Transcription

Several transcription factors are known which recognize specific response elements in the survivin gene promoter and are involved in the regulation of survivin mRNA transcription. Blocking transcription of the survivin gene through the inhibition of specific transcription factors seems to be an attractive concept to interfere with survivin function. YM155 (sepantronium bromide) was selected in a screen of a compound library as an inhibitor of a survivin promoter-reporter gene construct [46]. The compound was also able to suppress survivin expression in cultured cells and in transplanted PC-3 tumor cells in mice. This resulted in the inhibition of tumor growth. The promising animal experiments led to clinical studies in patients with advanced solid tumors in which favorable responses were observed [47]. The beneficial effects of YM155 were further underlined by enhancing the effects of docetaxel in malignant melanoma cells [48], by reversing the cis platinum resistance in head and neck cancer cells [49], by downregulating EGF receptor expression and its downstream effector pathways in pancreatic cancer cells [50], and by potentiating the function of the Bcl-2/Bcl-xL inhibitor ABT-737 in human glioma cells [51].

The mechanism of YM155 action in the negative regulation of the survivin gene has been the cause of a recent controversy. The induction of DNA damage by YM155 rather than the transcriptional repression has been proposed as the primary effect of the drug [52]. Additional evidence for a transcription-mediated mode of action, however, has come from experiments which showed that the survivin promoter is regulated by a complex of p54(nrb) and the transcription factor, interleukin enhancer-binding factor 3 (ILF3). p54(nrb) recognizes a specific sequence in the survivin promoter. YM155 binds directly to (ILF3)/NF110

[53], induces the disruption of the ILF3/p54(nrb) complex, and results in transcriptional silencing [54].

Additional transcription factors and inhibitory compounds have been used to interfere with survivin gene transcription. Tetra-O-methyl nordihydroguaiaretic acid (M4N) has been described as a transcriptional repressor of the survivin promoter. M4N is not survivin gene specific, but seems to repress genes dependent on the Sp1 transcription factor. M4N treatment of glioblastoma cells decreased the cell proliferation, enhanced the effects of the chemotherapeutic agent temozolomide (TMZ) and radiation, induced apoptotic cell death, decreased the mitotic index, and arrested the cell cycle in the G2/M phase [55].

Stat3 has been recognized as a potential therapeutic target for some time. This transcription factor regulates a number of transformation-associated target genes and its inhibition results in tumor cell death [56]. The small molecular weight inhibitor S3I-1757 is capable of disrupting Stat3 dimerization, prevents Stat3-mediated transactivation and suppresses the expression of target genes, e.g. survivin, but also Bcl-xL, cyclin D1, and MMP9 (matrix metallopeptidase 9). This results in the inhibition of tumor cell growth, migration, and invasion [57, 58]. The transcription factor Sox2 is also directly involved in the regulation of survivin gene transcription. Sox2 downregulation results in a decrease in survivin expression, caspase-9 activation, and initiation of mitochondria-dependent apoptosis. Agents interfering directly with Sox2 transactivation could therefore possibly become of interest as modulators of survivin expression [59].

Other compounds exerting their effects on survivin expression, indirectly by interference with transcription factors, are histone deacetylase (HDAC) inhibitors. Treatment of tumor cells with belinostat resulted in the downregulation of survivin on the mRNA and protein level, possibly through increased expression of transforming growth factor beta receptor II (TGFβRII) [60].

Finally, it has been shown that caspase-2 represses transcription of the survivin gene through the caspase-2-mediated proteolytic cleavage of the nuclear factor kappa B (NF-κB) activator, receptor-interacting protein 1 (RIP1). Degradation of RIP1 prevents transcription of NF-κB target genes, among them survivin. This counteracts NF-κB-dependent cell survival and results in deregulated mitotic transitions, enhanced apoptosis, and suppression of tumorigenicity [61].

4.2 Interference with Survivin Messenger RNA Utilization Through Antisense Oligonucleotides, Small Interfering RNA, and MicroRNA

Inhibition of specific transcription factors, as described above, can lead to a decrease in survivin mRNA and depletion of the survivin protein. A similar effect can also be achieved through interference with survivin mRNA utilization. Antisense oligonucleotides (ASOs), small interfering RNA (siRNA) and micro RNA (miRNA) can serve this purpose.

Oligonucleotides complementary with human survivin mRNA have been synthesized. These ASOs contain a phosphorothioate backbone and 2′-MOE (2′-O-methoxy ethyl/phosphorothioate) modifications of the ribose of the first four and last four nucleotides of the ASO, which enhances the affinity for target RNA, increases the plasma stability, and decreases toxicity when compared with earlier compounds. When ASOs are introduced into cells, they bind to their complementary target mRNA and cause their degradation through the activity of RNase H or they inhibit the mRNA utilization and translation into proteins. The survivin-directed ASO, LY2181308, a product of second-generation chemistry, potently inhibited expression of survivin when it was introduced into tumor cells. It caused the induction of caspase-3 activity and arrested cell division. LY2181308 was also active in vivo when administered intravenously in human xenograft mouse models. It inhibited tumor growth and enhanced the effects of gemcitabine, paclitaxel, and docetaxel [62].

EZO-3042 is another ASO directed against survivin. It is based on an engineered O2′ to C4′ linkage within the ribose sugar which locks the molecule in the 3′-endo structural conformation favoring RNA binding. This 16mer targets the region of the stop codon of the open reading frame and EZN-3042 introduction into cells resulted in a strong downregulation of survivin mRNA and growth inhibition of cultured tumor cells [63]. These ASOs are being evaluated in combination with chemotherapeutic drugs in clinical trials [24, 25].

Post-transcriptional gene silencing by RNAi is an effective tool to verify drug targets and will find its place in cancer therapy. siRNA is more effective than antisense RNA or ASO and has a remarkable target specificity. siRNA specific for survivin has been delivered into target cells in culture and has shown its effectiveness in a range of cancer cell lines from different indications [56, 64–66]. It not only caused polyploidy, growth arrest and apoptosis, but also increased the sensitivity of the cells towards chemotherapeutic agents. Delivery of survivin siRNA in vivo is still technically challenging, but the application of polyethylenimine (PEI)/siRNA complexes have been shown to be able to downregulate survivin in mice and have yielded promising therapeutic results in animals transplanted with glioma cells [64].

Survivin mRNA seems to be a target for miR-34a. miR-34a negatively regulates survivin protein expression and thus is able to inhibit gastric cancer cell proliferation and invasion. Silencing the survivin gene in tumor cells via the

upregulation of miR-34a might become a strategy of the future [67].

4.3 Interference with Signaling Pathways Regulating Survivin Gene Expression

Signaling pathways regulate cytoplasmic kinase cascades and transcription factor activities and subsequently change gene expression patterns. The signaling potential of multiple growth factors is mediated through effector molecules such as PI3K/AKT, extracellular signal-regulated protein kinases 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and JAK/Stat3, and governs survival and proliferation of normal and of tumor cells. Survivin is a target of EGF signaling in cancer cells. EGF-mediated induction of survivin requires the activity of Raf-1 and MEK/ERK, but EGF has no significant effect on survivin transcription. It prolongs the half-life of the survivin protein and stabilizes it by inhibiting survivin ubiquitination [68]. Many antibodies and small molecular-weight compounds aimed at members of the EGF receptor family and its downstream effectors are registered drugs or in clinical development [69]. These drugs might exert some of their functions through the destabilization of survivin.

A similar mechanism might be responsible for the high levels of survivin expression in human tumors harboring mutant K-Ras. Depletion of K-Ras in such tumor cells causes a decrease in survivin levels, following ubiquitination and proteasomal degradation of survivin, and impedes anchorage-independent growth, invasion, and survival of the tumor cells [70]. Inhibitors of downstream effectors of K-Ras might be able to induce similar phenotypes. The leukemic fusion protein BCR-ABL induces survivin expression at both the mRNA and protein levels and inhibits the apoptotic regulation in chronic myeloid leukemia cells. BCR-ABL-mediated upregulation of survivin requires JAK2/Stat3 activation [71]. Specific inhibitors for these signaling components are also available [19, 58]. Wogonin, a plant flavonoid compound, activates ERK and caspases in MCF-7 cells, but blocks the PI3K/Akt/survivin signal pathway, thus causing a proapoptotic effect [72].

Not all signaling pathways necessarily lead to the induction of survivin levels. The mTOR protein is being explored as a potential therapeutic target in cancer patients and this signaling molecule seems to negatively regulate survivin expression. The inhibition of mTOR with rapamycin in neuroblastoma cells led to an induction in survivin mRNA and protein levels and the protection of these cells against apoptosis. The beneficial and the counterproductive effects of mTOR inhibitors in particular tumor cell settings have to be carefully balanced [73].

4.4 Interference with Secondary Modifications of Survivin Regulating its Subcellular Locations and Distinct Functional Properties

The distinguishable functions of survivin in individual subcellular compartments are most likely realized through the domain structure of the molecule and regulated by secondary modifications. The modifying enzymes are potential targets for designed interference with distinct survivin functions, and survivin sequestration in particular subcellular compartments can possibly be exploited. Survivin is present in the cytoplasm, the mitochondria, and the nucleus. It performs distinct functions in these subcellular compartments. Survivin can inhibit both the extrinsic and the intrinsic pathways of apoptosis induction by interference with the activation of caspases in the cytoplasm and in mitochondria. In the nucleus, it can form a complex with chromosomal passenger proteins, e.g., Aurora-B kinase, inner centromere protein (INCENP), and Borealin to regulate cell division. The particular subcellular location and the assumption of specific functional properties are regulated by the domain structure and signal-dependent secondary modifications of the protein. Survivin is phosphorylated at threonine 117 by the Aurora-B kinase. This directs survivin to the centromere and regulates the assembly of the chromosomal passenger complex. Aurora-B kinase activation is preceded by phosphorylation at serine 20, which is catalyzed by PLK-1, and both phosphorylation events are required for the correct spindle microtubule attachment. Phosphorylation at threonine 34 is critical for the anti-apoptotic function of survivin [74].

The nuclear accumulation of survivin is dependent upon its acetylation on lysine 129 by cyclic adenosine monophosphate response element-binding (CREB)-binding protein (CBP). Survivin acetylation results in its homodimerization. The non-acetylated form of survivin heterodimerizes with CRM1, and thus becomes destined for nuclear export [35].

HDAC6 is responsible for reversing CBP-mediated survivin acetylation. HDAC6 binds to and deacetylates survivin. The relative concentrations of acetylated and non-acetylation survivin at lysine 129 determine its interaction with CRM1, and thus regulate the nuclear export of survivin [75]. Nuclear survivin also interacts with Stat3 and represses the Stat3 transactivation potential [35]. The modifying enzymes are potential targets for designed interference with the diverse survivin functions. The sequestration in particular subcellular compartments can be envisaged [76].

4.5 Interference with Survivin Folding, Stability, and Interaction Partners

Heat shock proteins (HSPs) are molecular chaperones that assist in the folding and the assumption of a stable

conformation of proteins. They prevent the formation of protein aggregates. HSPs are often over-expressed in tumor cells. HSP90 is a member of this gene family and an essential adenosine triphosphatase-dependent molecular chaperone. It assists in protein folding and quality control, maturation of client proteins, and protein trafficking among subcellular compartments. It is also involved in the stabilization of client proteins which regulate apoptosis, proliferation, autophagy, and cell cycle progression. Inhibitors of HSP90 have been developed that can simultaneously modulate several intracellular regulatory pathways [77]. Because of its restricted repertoire of client proteins, typically kinases and signaling molecules, HSP90 occupies a unique position in cellular homeostasis [78].

Survivin is a HSP90 client protein and the interaction domains between the two proteins have been exploited to derive inhibitory molecules. Shepherdin is an oligopeptide that comprises the survivin sequences from lysine 79 to glycine 83, coupled to a protein transduction sequence allowing the uptake of the molecule into cells. Shepherdin binds to HSP90, inhibits the formation of the survivin HSP90 complex, and competes with adenosine triphosphate binding to HSP90. Upon its uptake into, for example, acute myeloid leukemia cells, it induced cell death in a large fraction of the transduced cells. It also inhibited the growth of transplanted tumor cells in mice [79]. Treatment of glioblastoma cells with shepherdin caused the irreversible collapse of mitochondria, degradation of HSP90 client proteins in the cytosol, and tumor cell killing. Targeting the HSP90 functions in different subcellular compartments could become therapeutically beneficial, at least partly through its effects on survivin action [80].

4.6 Induction of Immune Responses Against Survivin-Expressing Cells

Progress in understanding the molecular basis of cancer etiology and insights into immunologic defense mechanisms have led to promising new treatment options for cancer patients [81, 82]. The enhancement of the immune system has been validated as a promising therapeutic strategy to elicit tumor-specific responses, induce durable tumor regression, and improve survival intervals of patients [83]. Tumor cells are poor antigen-presenting cells and the induction of protective immunity depends upon efficient tumor antigen presentation by activated dendritic cells. They display surface antigens via major histocompatibility complex class I and II in combination with co-stimulatory molecules, e.g. B7-1 and B7-2, and are able to interact with naïve CD4+ and CD8+ T-cells to trigger T-cell proliferation and differentiation. Differentiated cytolytic CD8+ T-lymphocytes (CTLs) are the most important effector cells for anti-tumor immune responses. Survivin is a tumor-

associated antigen (TAA) and therefore a potential target for immunotherapy. Its immunogenicity was indicated by the presence of survivin-specific CD8+ T-cells and survivin-specific IgG antibodies in cancer patients [84], and HLA class II restricted epitopes have been identified. Survivin-derived epitopes have been used in vaccination experiments to activate CD4+ responses in prostate cancer [85] and melanoma patients [86]. Survivin-specific T-cell activities were induced, which correlate with tumor response and patient survival.

4.7 Interference with Survivin Function Through Interacting Peptide Ligands

Survivin has been validated as a drug target by the approaches described above [23–25, 87]. In particular, the downregulation of survivin mRNA expression in tumor cells with a lentiviral gene transfer vector encoding a specific small hairpin RNA (shRNA) seems most convincing [26]. Survivin has a favorable, tumor-preferential expression profile, but it is a difficult drug target. Unconventional approaches have been used to exploit its expression in tumor cells for therapeutic purposes. These include the interference with survivin gene expression at the transcriptional level, the inhibition of survivin mRNA translation with ASOs and siRNA, the interference with survivin functions through dominant negative variants of the molecule and peptide antagonists, DNA vaccines and immunotherapeutic strategies, and the use of small molecular-weight compounds that target protein interactions [30, 33, 60, 88, 89]. These strategies are based on indirect mechanisms. They affect the expression and eventually the function of the protein, but they are not based on the direct binding of a drug to survivin.

An alternative strategy has recently been described which exploits survivin-specific ligands to derive an inhibiting molecule. The strategy employs the screening of a complementary DNA (cDNA) library for surviving interacting proteins in a yeast two hybrid setting. Several survivin interaction partners have previously been described, e.g. the pro-apoptotic protein Smac [88] and the borealin component of the chromosomal passenger complex [90], but a novel interacting protein, a domain of the ferritin heavy chain 1 (FTH1) has been detected [26]. Ferritin is a widely expressed protein and can sequester free intracellular iron. FTH1 embodies ferroxidase activity and can convert Fe^{2+} to the less reactive and less toxic Fe^{3+}. This function involves the coordinated activity of ferritin heavy chain (FTH1) and ferritin light chain subunits.

The survivin-interacting domain of FTH1 has been integrated 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. Introduction of the purified protein into survivin over-expressing breast cancer and glioma cells, cells which express high levels of survivin, resulted in survivin downregulation, decreased the growth and viability of tumor cells in culture, and reduced growth of the cancer cells upon transplantation into immunodeficient mice. rSip selectively targets the anti-apoptotic function of survivin and causes tumor cell death. The effects of shSurvivin-induced downregulation of its mRNA or the interference with survivin function by rSip are remarkably specific to tumor cells. No growth inhibition and induction of apoptosis were observed in non-transformed MCF-10A and NIH/3T3 cells, they remained unaffected [26]. rSip, as a peptide ligand of survivin, provides a lead structure for the development of drugs targeting the tumor cell "addiction protein" survivin.

The use of peptides to affect the induction of apoptosis has previously been shown [91]. Peptides derived from the pro-apoptotic Smac protein, another member of the IAP family, are able to inhibit XIAP. This protein blocks caspase activity and is over-expressed in cancer cells [92, 93]. Smac peptides, transduced into cells, induced tumor cell apoptosis by inhibition of XIAP and the subsequent activation of caspases. Smac-TAT-peptides acted synergistically with TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), FasL, doxorubicin, or cisplatin in the induction of apoptosis in malignant glioma cells [94]. The experiments show that apoptosis induction in cancer cells, dependent upon the over-expression of antiapoptotic proteins such as XIAP or survivin, can be initiated by specifically interacting peptides, e.g., Smac-derived peptides or rSip.

5 Prospective Tasks: Derivation of Small Molecular-Weight Compounds from Peptide Ligands

Cell culture experiments have convincingly shown that peptides and recombinant proteins have the ability to enter cells and inhibit target protein functions through specific binding interactions. These cell culture experiments, however, cannot easily be extrapolated to the in vivo situation. Biologic macromolecules, nucleic acids, and polypeptides have intrinsic properties that are unfavorable for their use as systemically applied drugs [95]. Limitations arise from their solubility, stability, toxicity, and ability to cross cell membranes. The short half-life of the recombinant proteins in the circulation of mice, often less than 10 min, precludes that a lasting target inhibition, sufficient to exert, for example, growth inhibitory and apoptotic effects. Experiments have been carried out with intracellularly acting peptides administered in a systemic fashion, and limited

therapeutic effects have been observed [96]. The dominant negative survivin T34A mutant was produced as a recombinant protein coupled to a protein transduction domain and was found to be able to reduce melanoma cell growth in experimental animals [97]. The growth of transplanted glioma cells could only be partially inhibited with a peptide targeting Stat3 [56].

For these reasons it seems reasonable to replace the peptides by low molecular-weight compounds that exert the same functional effects but exhibit better pharmacokinetic properties and bioavailability [20]. The peptides can serve as tools in screening procedures designed to identify such functional analogs. The screen can be based, for example, on the disruption of the binding between survivin and the FTH fragment. The availability of a peptide sequence that functionally inactivates a crucial domain of survivin can be exploited [26]. The peptide sequence can serve as a tool in screening procedures that allow the identification of small molecular-weight compounds, which are functionally equivalent to the FTH1-derived peptide sequence [98, 99]. The discovery of low molecular-weight inhibitors of peptide–protein or PPIs is a most important issue in modern drug development, one not restricted to survivin research [100].

The procedure comprises the steps schematically depicted in Fig. 2. Peptide sequences, which very specifically interact with essential domains of target proteins, can be identified in yeast two hybrid screens [101]. Random peptide libraries of high complexity can be employed and peptide ligands for target domains can be identified regardless of structural considerations. The mere interaction between the peptide ligand and the bait domain of the target protein does not make predictions about the functional consequences of the binding reaction. The peptide sequences, however, can be validated as functional inhibitors upon expression in cells or by exogenous transduction into cells mediated by protein transduction domains. This requires that the inhibition of the target protein triggers a robust, recognizable cellular phenotype [102, 103].

In a next step, the peptide ligand serves as a tool in a high-throughput screen approach and the selection of suitable molecules from a low molecular-weight compound library. The Alpha Screen®, an amplified luminescent proximity homogeneous assay, is based on fluorescence resonance and used to detect the interaction of two molecules [104]. For this purpose, each one of the interaction partners is being conjugated with dextran or hydrogel beads that 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

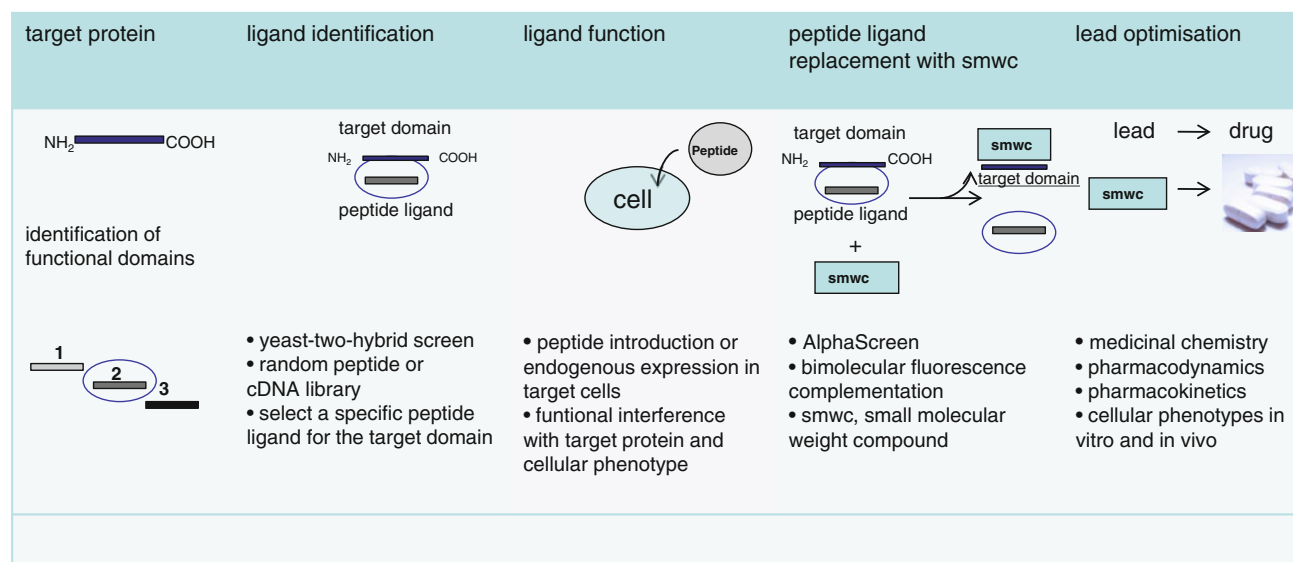## Peptide ligands and derivation of lead compounds for drug development

Fig. 2 Peptide ligands and derivation of lead compounds for drug development. Many proteins are structured in distinct functional domains. Genetic analysis can reveal domains indispensable for, for example, the growth and survival of cancer cells. Specific peptide ligands able to recognize and suppress the functions of such crucial domains and interaction surfaces can be derived through screening assays in yeast cells. The peptides can be present in random peptide libraries or cDNA libraries [101]. The interaction partners can be functionally evaluated, they should bind and inhibit their target proteins and, for example, should be able to induce cancer cell death [111]. However, further technology development is required to turn such ligands into useful drugs [20]. The technology comprises three steps: (1) identification of a peptide ligand that specifically interacts with a crucial functional domain of a target protein; (2) verification of the functional consequences of ligand binding, e.g., induction of a desired cellular phenotype upon intracellular interaction of the peptide ligand with its target structure; and (3) replacement of the peptide ligand with a functionally equivalent low molecular-weight, drug-like compound and its optimization through medicinal chemistry. *cDNA* complementary DNA

reactive singlet oxygen, and a fluorescent signal of 520–620 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 system can be formatted for high throughput and complex compound libraries can be screened. The compounds obtained in these in vitro assays can be further investigated and their usefulness as drugs can be assessed in cell culture. For this purpose, a related technology, BiFC (bimolecular fluorescence complementation) can be used. It 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. Interference with BiFC is a method that allows the detection of the disruption of an intracellular protein interaction, a situation most favorable and suited for drug discovery and development [105].

The identification of drug-like molecules from interacting peptide sequences in appropriate screening assays could lead to a significant extension of useful drug targets and the discovery of suitable lead compounds.

6 Discussion

A large number of molecules have been identified that are functionally involved in the etiology and progression of cancer and which could serve as potential drug targets [106]. The most promising among them are proteins that 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 [107]. 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. Proteins that do not exhibit these features are usually considered as non-druggable. The development of technologies that would allow the exploitation of the large repertoire of molecules with crucial functional roles in pathologic processes, but suboptimal characteristics of conventional drug targets, would be of great value [108].

Survivin belongs to this class of drug targets. They are obviously promising, but it is difficult to derive inhibitory compounds against these targets. Survivin is an essential component of multiple functional protein complexes in cancer cells. It serves in collaboration with other proteins in, for example, cell division, the regulation of apoptosis, and cell motility. Since no small molecular-weight ligands for survivin are known that could be used as lead compounds, indirect strategies have been pursued. Insights into the complex regulation of its expression, on the transcriptional and post-transcriptional level, the secondary modifications governing its subcellular location, protein interactions and immunogenic properties have been considered and exploited as targets. The inhibition of signaling pathways, transcription factors or protein modifying enzymes, however, invariably has pleiotropic effects. The side effects of such therapeutic agents are difficult or impossible to predict. The use of drugs with a defined target specificity, acting exclusively on the level of the survivin protein, is therefore preferable. Specific peptide ligands, able to recognize and suppress the functions of crucial interaction surfaces of survivin, could pave the way. One such peptide has been derived and shown to be able to inhibit survivin function and induce cancer cell death. However, similar to other biological macromolecules with intracellular sites of action, these peptides are suboptimal compounds when they are delivered systemically. The technology to turn such peptide ligands into useful drugs is being developed. They involve sequential screening procedures: first the identification of an inhibitory peptide ligand, and subsequently the conversion of such a peptide into a functionally equivalent small molecular-weight compound. These strategies could result in a large extension of new drug targets and, more importantly, in a plethora of beneficial drugs.

Constitutively activated signaling molecules have been identified as drivers of cellular transformation and are the favorite targets for therapeutics [69]. However, the inhibition of such molecules is often counterbalanced by pathway interconnections which result in adaptive resistance and limited therapeutic responses [109, 110]. The identification and inhibition of targets that are unable to elicit adaptive responses seems crucial to achieve durable clinical benefits. Survivin might well belong in this category.

7 Conclusions

Survivin is distinguished by several functional properties which are desirable for promising cancer drug targets. Its expression occurs preferentially in cancer cells, but is hardly detectable in normal tissues. It assumes functions in different subcellular compartments and participates in the control of cell division, apoptosis, the cellular stress response, the regulation of cell migration, and metastasis formation. Survivin expression serves as a biomarker, high expression indicates an unfavorable prognosis and resistance to chemotherapeutic agents and radiation treatment. Despite these favorable properties, it has been difficult to exploit the molecule as a drug target. The structure of survivin does not reveal hydrophobic binding pockets that could serve as docking sites for low molecular-weight ligands. Several indirect approaches have been exploited to affect its function and influence the phenotype of survivin-expressing cells. Interference with the expression of the survivin gene, the utilization of its mRNA, the intracellular localization, the interaction with binding partners, the stability of the survivin protein, and the induction of survivin specific immune responses have been experimentally explored. A direct strategy to inhibit survivin has been recently pioneered, based on the identification of a specifically interacting peptide. This peptide can recognize survivin intracellularly and cause the degradation of the peptide ligand–survivin complex. Technology is being developed which utilizes the peptide ligand–survivin interaction in high-throughput screening assays and which might yield small-molecular weight, drug-like compounds with functional properties of survivin inhibitors.

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