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Survivin as a target for new anticancer interventions

Nadia Zaffaroni *, Marzia Pennati, Maria Grazia Daidone

Dipartimento di Oncologia Sperimentale, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy

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Abstract

Survivin is a member of the inhibitor of apoptosis protein (IAP) family, that has been implicated in both control of cell division and inhibition of apoptosis. Specifically, its anti-apoptotic function seems to be related to the ability to directly or indirectly inhibit caspases. Survivin is selectively expressed in the most common human neoplasms and appears to be involved in tumor cell resistance to some anticancer agents and ionizing radiation. On the basis of these findings survivin has been proposed as an attractive target for new anticancer interventions. Several preclinical studies have demonstrated that down-regulation of survivin expression/function, accomplished through the use of antisense oligonucleotides, dominant negative mutants, ribozymes, small interfering RNAs and cyclin-dependent kinase inhibitors, increased the apoptotic rate, reduced tumor-growth potential and sensitized tumor cells to chemotherapeutic drugs with different action mechanisms and γ-irradiation in *in vitro* and *in vivo* models of different human tumor types.

Keywords: Survivin • apoptosis • anticancer drugs • ionizing radiation • antisense oligonucleotides • dominant negative mutants • ribozymes • small interfering RNAs • cycle-dependent kinase inhibitors

Introduction

Defects in the physiological pathways for apoptosis contribute to many diseases including cancer [1]. Moreover, impairment of apoptotic cell death leads to treatment resistance of cancer cells [2, 3].

Apoptosis is mediated by caspases, a family of cysteine proteases that become activated by proteolysis and cleave multiple cellular substrates. Two major pathways of apoptosis have been identified in mammalian cells. An "extrinsic" pathway is triggered by the binding of ligands [4] to cell-surface trimeric membrane death receptors and leads to caspase-8 activation (Fig. 1). An "intrinsic" apoptotic pathway involves mitochondria, which respond to pro-apoptotic signals by releasing

* Correspondence to: Nadia ZAFFARONI, PhD,
Dipartimento di Oncologia Sperimentale Unità Operativa 10
Istituto Nazionale per lo Studio e la Cura dei Tumori
Via Venezian 1, 20133 Milano, Italy.
Tel.: +39 02 2390 3260; Fax: +39 02 2390 3052
E-mail: nadia.zaffaroni@istitutotumori.mi.it

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cytochrome *c*. Cytochrome *c* binds and activates the Apoptotic Protease Activating Factor-1 (Apaf-1), causing assembly of a multiprotein caspase-activating complex (apoptosome) and leading to activation of caspase-9 and initiation of a protease cascade [5] (Fig. 1). This pathway is primarily governed by proteins of the Bcl-2 family, which include anti-apoptotic molecules and pro-apoptotic molecules able to differentially affect mitochondrial homeostasis and cytochrome c release [6]. Moreover, other proteins belonging to the inhibitor of apoptosis protein (IAP) family, including ML-IAP, XIAP, $cIAP_1$, $cIAP_2$, NIAP, apollon and survivin, are able to block a common step downstream of mitochondrial cytochrome *c* release by inhibiting terminal effector caspase-3 and caspase-7, and interfering with caspase-9 activity and processing [7].

Survivin structure and function

The human survivin gene spans 14.7 kb on the telomeric position of chromosome 17 and is localised to band q25 [8]. It comprises three introns and four exons, a TATA-less proximal promoter, and approximately 200 nt GC-rich regions upstream of exon 1 [9]. The gene encodes a 16.5 kD protein of 142 amino acids. Structurally, it is composed of a single Baculovirus IAP Repeat (BIR) domain and an extended COOH-terminal α -helical coiled-coil domain [10]. Moreover, it does not contain a RING-finger domain, found in other IAPs. Splicing variants of survivin have been identified. Survivin-2B is generated by insertion of an alternative exon, survivin-ΔEx3 arises from the removal of the exon 3, and survivin-3B results from the introduction of a novel exon 3B [11, 12]. Very recently, an additional splice variant, Survivin 2alpha, has been identified. Structurally, the transcript consists of 2 exons: exon 1 and exon 2, as well as a 3' 197 bp region of intron 2. Acquisition of a new in-frame stop codon within intron 2 results in an open reading frame of 225 nucleotides, predicting a truncated 74 amino acid protein [13]. Little is known about the differential functions of survivin alternative splice forms. However, preliminary data would suggest that heterodimerization of survivin with survivin-ΔEx3 is essential for the inhibition of mitochondrial-dependent apoptosis [14]. Moreover, it has been demonstrated in exogenous expression assays that survivin 2alpha attenuates the anti-apoptotic activity of survivin [13].

Survivin is regulated in a highly cell cycle-dependent manner, with a marked increase in the G_2M phase [15]. During this phase survivin associates with and is phosphorylated by $p34^{cdc2}$/cyclin B1 kinase [16]. It has been demonstrated that survivin exists in two immunohistochemically distinct pools, with a nuclear pool localised to kinetochores of metaphase chromosomes and to the central spindle midzone at anaphase, and a cytosolic pool associated with interphase microtubules, centrosomes, spindle poles and mitotic spindle microtubules at metaphase and anaphase [17]. However, the microtubule-associated pool appears to be quantitatively predominant and functionally relevant. These findings, together with the phenotype of knockout mice (which is characterised by a catastrophic defect of microtubule assembly, with absence of mitotic spindle, formation of multinucleated cells and 100% embryonic lethality [18]), are consistent with a critical role of survivin in mitosis to preserve the mitotic apparatus and to allow normal mitotic progression. In fact, it has been demonstrated that survivin down-regulation causes pleiotropic cell-division defects [19, 20]. Moreover, Giodini *et al.* [21] showed that forced expression of survivin in HeLa epithelial carcinoma cells profoundly influenced microtubule dynamics and also caused stabilization of microtubules against nocodazole-induced depolymerization, thus indicating that survivin may facilitate evasion from checkpoint mechanisms of growth arrest and, consequently, promote resistance to drugs targeting the mitotic spindle. Additional evidence indicates that survivin also participates in the regulation of chromosome segregation [22], and that the protein cooperates together with the chromosomal passenger proteins INCENP and Aurora-B to perform its mitotic duties [23].

The existence of a mitochondrial pool of survivin, which is able to orchestrate a novel pathway of apoptosis inhibition in tumor cells, has recently been reported [24]. Specifically, it was found that, in response to cell death stimulation,

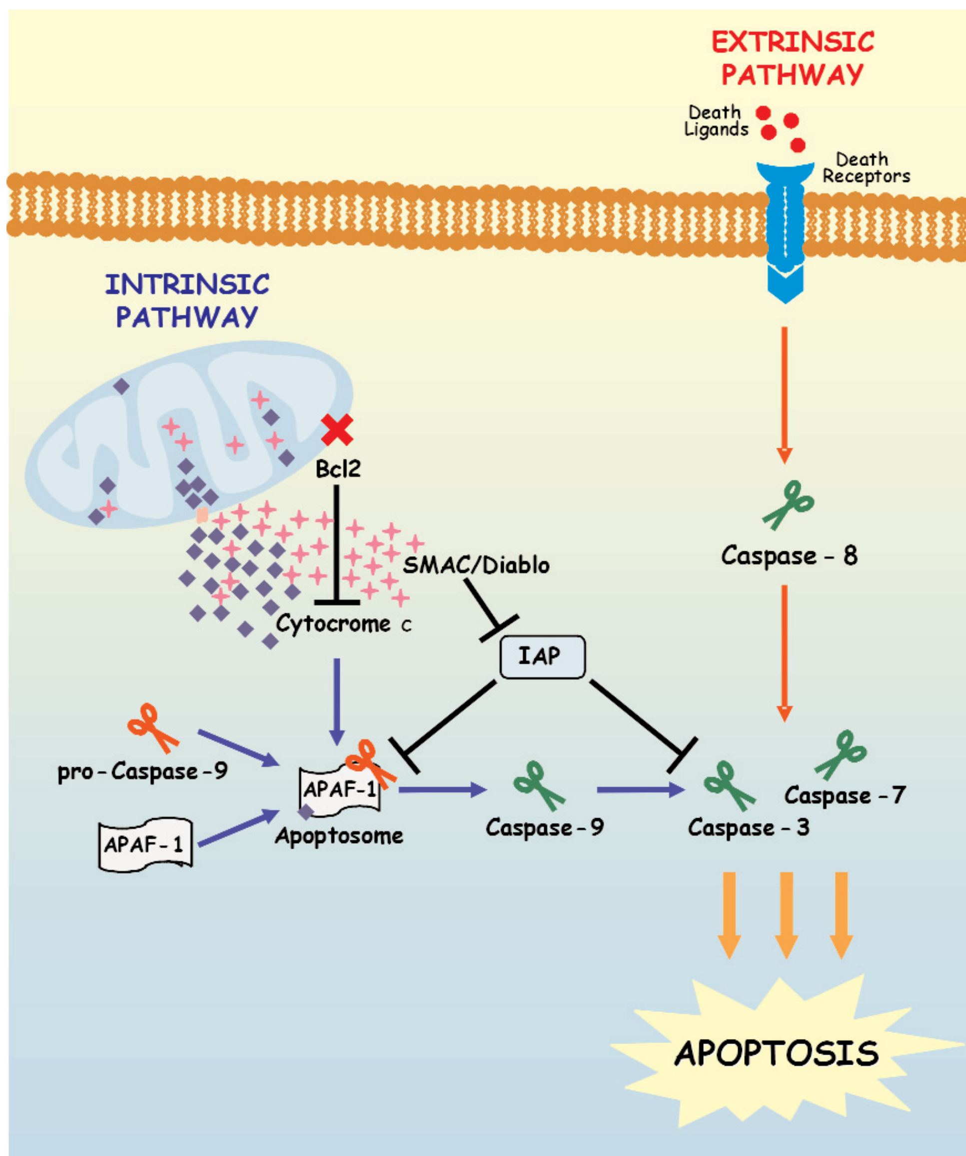

Fig. 1 Schematic representation of the two major apoptotic pathways in human cells: the "extrinsic" (the death receptor-mediated) and "intrinsic" (mitochondrial) programmed cell death.

mitochondrial survivin is rapidly discharged in the cytosol, where it prevents caspase activation and inhibits apoptosis.

It has been demonstrated that Hsp90, a molecular chaperone that is the central regulator of cellular stress response, associates with survivin. Such a physical interaction, which involves the Hsp90 ATPase domain and the survivin BIR domain, is required for survivin stability and function. In fact, targeted antibody-mediated disruption of the survivin-Hsp90 complex in cancer cells resulted in proteasomal degradation of survivin, mitochondrial-dependent apoptosis and mitotic arrest [25].

Survivin expression in human tumors

Survivin is strongly expressed in embryonic and fetal organs but has not been reported in differentiated normal tissues with the exception of thymus, basal colonic epithelium [26] endothelial cells and neural stem cells during angiogenesis [27]. Several reports have demonstrated survivin expression in the majority of human tumor types including lung, breast, colon, gastric, oesophageal, pancreatic, liver, bladder, uterine and ovarian cancers, large-cell non-Hodgkin's lymphomas, leukaemias, neuroblastoma, brain tumors, pheochromocytoma, soft

tissue sarcomas, melanomas and other skin cancers [26]. Moreover, the expression of survivin has been also detected in a variety of preneoplastic and/or benign lesions including polyps of the colon, breast adenomas, Bowen's disease and hypertrophic actinic keratosis [26], suggesting that re-expression of survivin may occur early during malignant transformation or following disturbance in the balance between cell proliferation and death. The up-regulation of survivin at the transcriptional level in human tumors has been confirmed in genome-wide searches, which indicated survivin as the fourth top "transcriptome" in cancers of various histology [28]. At least for some tumors types molecular abnormalities have been described that may account for the increased expression of survivin in cancer compared to normal tissue. Specifically, in neuroblastoma a gain of 17q25 containing the survivin locus represents a frequent genetic abnormality [29]. Moreover, in most ovarian cancers survivin exon 1, which is silenced by methylation in normal ovarian epithelium, becomes unmethylated and, consequently, transcriptionally active [30]. Survivin overexpression in tumors has been recently linked to loss of wild-type p53 [31]. Specifically, it was seen that accumulation of wild-type p53 in human ovarian cancer cells induced survivin transcriptional repression which did not require direct sequence-specific DNA binding of p53 to the survivin promoter. Modifications of chromatin structure within the promoter could be the molecular explanation for silencing of the survivin gene by wild-type p53.

In the majority of solid tumors investigated for survivin expression (including breast, lung, colorectal, gastric, liver, bladder and kidney cancers, neuroblastoma, gliomas and soft tissue sarcomas), high levels of the protein were predictive of tumor progression, either in terms of disease-free survival or overall survival, thus providing prognostically relevant informations [26]. As regards hematological malignancies, a significantly shorter survival was observed in large B-cell lymphoma [32] as well as mantle cell lymphoma [33] patients with high survivin expression. Moreover, survivin proved to be a negative prognostic factor in patients with acute myeloid leukemia [34]. In several neoplasms, the association with tumor progression has been also corroborated in the context of comprehensive analysis of gene-expression profiling by DNA microarray or PCR-based assays.

Survivin expression and resistance to anticancer agents

Considering that apoptosis is the primary mode of cell death induced by several classes of anticancer agents and ionizing radiation, a possible general role of survivin in determining the chemo- and radio-sensitivity profiles of tumor cells has been hypothesised. Moreover, since survivin is associated with microtubules and with the mitotic spindle it is likely that this protein can specifically contribute to the response of cells to microtubule-interacting agents.

Li *et al.* [35] first demonstrated that transfection of wild-type survivin efficiently protected murine NIH3T3 fibroblasts from apoptosis induced by the microtubule-stabilising agent taxol. In agreement with this observation, Giodini *et al.* [21] reported that infection of HeLa cells with an adenoviral vector expressing survivin suppressed apoptosis induced by taxol. Based on this finding, our laboratory performed a parallel investigation on cell lines and clinical specimens from ovarian carcinomas to determine whether survivin is involved in regulating cell sensitivity to taxanes. The OAW42 and IGROV-1 human ovarian cancer cell lines were transfected with the human survivin cDNA. Stable transfection with survivin cDNA was able to protect these cells from the cytotoxic effects induced by taxol and taxotere, with IC_{50} values for the survivin-transfectant cell populations 4-6-fold those of the control cells [36]. It has been reported that taxol-induced microtubule stabilization and mitotic arrest increase survivin expression, which engenders a cell survival pathway to counteract taxol-induced apoptosis [37]. However, it does not seem that this mitotic survival pathway is the only one by which cancer cells counteract taxol-induced programmed cell death. In fact, Ling *et al.* [38] recently reported that induction of survivin by taxol in MCF-7 cells is an early event and is independent of taxol-mediated G_2/M arrest, thus suggesting a role for survivin in taxol resistance not only during mitosis but outside of the mitotic checkpoint as well.

In the clinical setting, when we analyzed the response of 95 advanced ovarian cancer patients to a taxol/platinum-based regimen as a function of survivin expression, we found a significantly higher clinical or pathologic response rate in

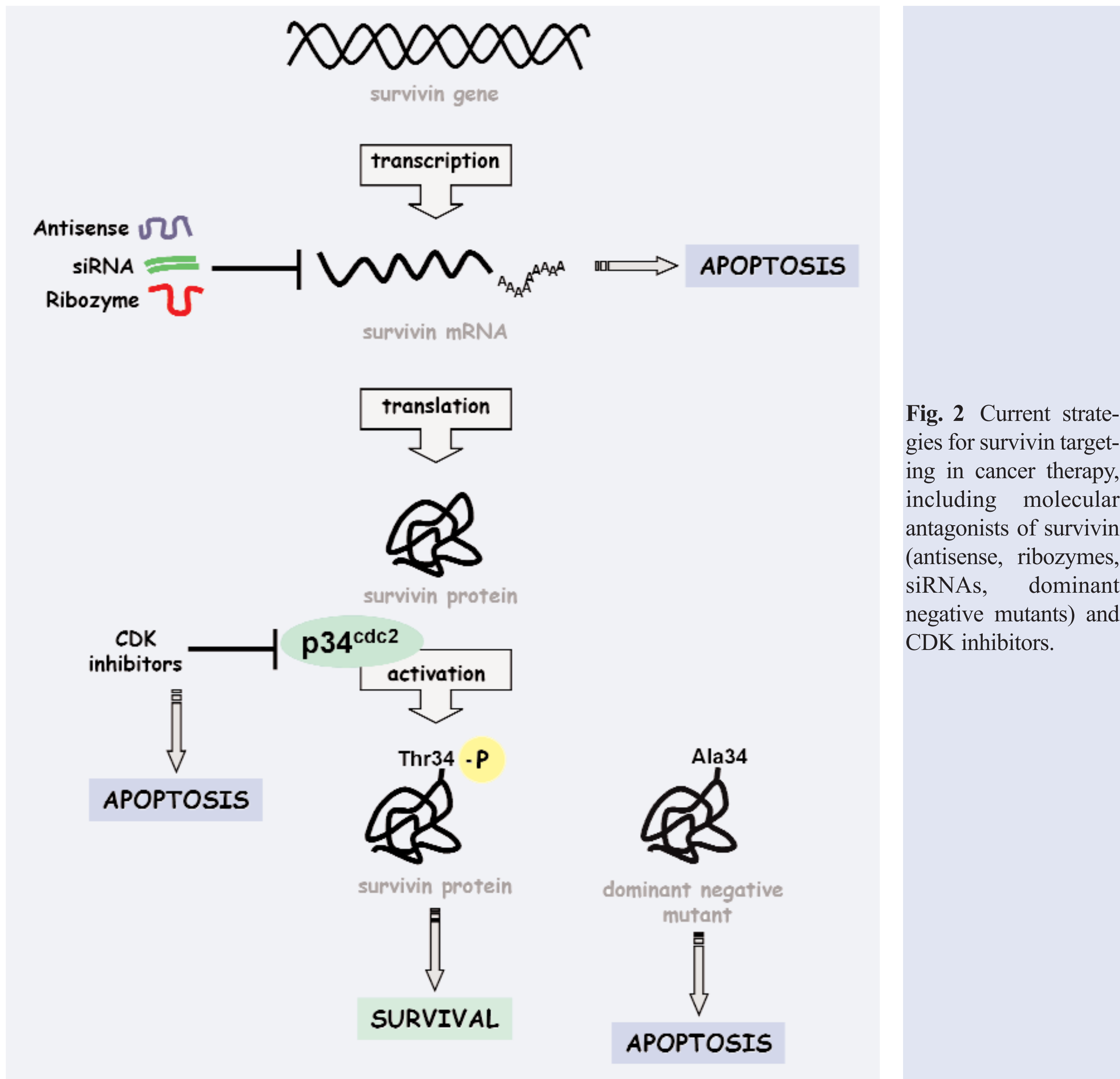

Fig. 2 Current strategies for survivin targeting in cancer therapy, including molecular antagonists of survivin (antisense, ribozymes, siRNAs, dominant negative mutants) and CDK inhibitors.

cases with absent/low protein expression than in those expressing high levels of survivin (75% vs 43%) [36].

Regarding the possible role of survivin in determining the radiation response of human tumor cells, Asanuma *et al.* [39] reported that survivin acts as a constitutive radio-resistance factor in pancreatic cancer cells. Specifically, in a panel of established cell lines they found an inverse relationship between survivin mRNA expression and *in vitro* sensitivity to X-irradiation. Moreover, these authors also demonstrated that survivin mRNA expression was increased by sublethal doses of X-irradiation, which would suggest that the protein also acts as an inducible radio-resistance factor.

Very recently, Zhang *et al.* [40] showed that survivin mediates resistance to antiandrogen therapy with flutamide in prostate cancer cells. Specifically, these authors suggested that upregulation of survivin *via* insulin-like growth factor-1/AKT signalling during androgen blockade may be one of the mechanisms by which prostate cancer cells develop resistance to antiandrogens.

Overall, the results obtained in the different studies indicate survivin to be a cellular factor potentially involved in the chemo-resistant and radio-resistant phenotypes of human tumors cells and suggest that

approaches designed to inhibit survivin expression may lead to human tumor sensitisation to chemical and physical agents.

Survivin as a target for new anticancer strategies

In recent years considerable efforts have been made by researchers to develop strategies for modulating apoptosis in cancer and other human diseases [41]. In this context, approaches to counteract survivin in tumor cells have been proposed with the dual aim to inhibit tumor growth through an increase in spontaneous apoptosis, and to enhance tumor cell response to apoptosis-inducing agents [42]. Different kinds of survivin molecular antagonists, including antisense oligonucleotides, ribozymes, small interfering RNAs (siRNAs) and dominant-negative mutants, as well as cyclin-dependent kinase inhibitors have bene used (Fig. 2; Tables I, II).

Antisense oligonucleotides

Grossman *et al.* [43] demonstrated that transfection of survivin antisense triggered spontaneous apoptosis in the absence of other stimuli in YUSAC2 and LOX human melanoma cell lines. Successively, Olie *et al.* [44] assessed the ability of a series of 20-mer phosphorothioate oligonucleotides directed against different sites within survivin mRNA to down-regulate the expression of the gene. They reported that the antisense oligonucleotide 4003, which targets nucleotides 232-251, reduced the abundance of survivin mRNA in the A549 human lung adenocarcinoma cell line in a dose-dependent manner with an IC50 around 200 nM. The antisense oligonucleotide also inhibited A549 cell growth in a dose-dependent manner and induced apoptosis. Specifically, 4003-treated cells contained nuclei with condensed and fragmented chromatin and were characterised by an increased caspase-3 protease activity compared to parental cells. Moreover, chemosensitivity experiments showed a significantly higher rate of apoptosis in cells simultaneously exposed to the antisense oligonucleotide and the anticancer drug etoposide than in cells treated with either oligonucleotide or etoposide alone, thus indicating that down-regulation of survivin has the potential to sensitise A549 cells to the effects of the topoisomerase II inhibitor. Shankar *et al.* [45] used different antisense oligonucleotides spanning the survivin gene to inhibit the expression of survivin in neural tumors. Results obtained in this study suggest that survivin inhibition induces cell death through caspase-independent and caspase-dependent pathways in these tumor models. Specifically, the authors demonstrated that transfection of MSN human neuroblastoma cells with 20-mer phosphorothioate oligomers caused cell death in the absence of caspase activation. In contrast, TC620 oligodendroglioma cells exposed to the antisense oligonucleotides underwent caspase-dependent apoptotic cell death. Induction of apoptosis as a consequence of antisense-mediated survivin down-regulation was also observed by Xia *et al.* [46] in malignant pleural mesothelioma cell lines. More recently, Lu *et al.* [47] showed that inhibition of survivin expression accomplished through the use of antisense oligonucleotides was able to increase the sensitivity of H460 lung cancer cells to ionizing radiation.

Kanwar *et al.* [48] demonstrated that survivin inhibition resulted in increased sensitivity to immunotherapy in murine EL-4 thymic lymphoma. Specifically, tumors injected with plasmids encoding antisense survivin or the Cys84Ala survivin dominant negative mutant were significantly inhibited in their growth and showed an increased level of apoptosis compared to control tumors. Moreover, tumor growth was further reduced by concomitant injection of the T-cell costimulator B7-1, suggesting that survivin down-regulation sensitises lymphoma cells to B7-1-mediated immunotherapy.

Hammerhead ribozymes

As an alternative strategy for survivin inhibition we developed in our laboratory ribozymes directed against different portions of survivin mRNA. Ribozymes are small RNA molecules that possess specific endonucleolytic activity and catalyse the hydrolysis of specific phosphodiester bonds,

Table 1 Therapeutic strategies targeted to survivin

Experimental Approach	System	Biological Effects	Ref.
Antisense Oligonucleotides			
Natural antisense (EPR-1 cDNA)	Epithelial carcinoma and Melanoma cell lines	↓ proliferation ↑ spontaneous apoptosis	[9, 40]
Oligonucleotide 4003	Lung adenocarcinoma cell line	↓ proliferation ↑ spontaneous apoptosis ↑etoposide-induced apoptosis	[41]
Oligonucleotides 903 and 904	Neuroblastoma and Oligodendroglioma cell lines	↓ proliferation ↑ spontaneous apoptosis	[42]
Oligonucleotide	Pleural mesothelioma cell lines	↑ spontaneous apoptosis	[43]
Oligonucleotide	Lung cancer cell lines	↑ radiation-induced apoptosis	[44]
Oligonucleotide	Murine thymic lymphoma	↓ tumor growth ↑ spontaneous apoptosis ↑ sensitivity to immunotherapy	[45]
Hammerhead Ribozymes			
Hammerhead ribozymes	Melanoma cell lines	↑ cisplatin-induced apoptosis ↑ topotecan-induced apoptosis ↑ radiation-induced apoptosis	[50–52]
Hammerhead ribozymes	Prostate cancer cell lines	↓ proliferation ↑ spontaneous apoptosis ↑ cisplatin-induced apoptosis ↓ *de novo* tumor formation	[53]
Hammerhead ribozymes	Breast cancer cell line	↑ etoposide-induced apoptosis	[54]
siRNA			
siRNA	Cervical carcinoma cell line	↓ proliferation Cell cycle dysregulation	[24]
siRNAs	Sarcoma cell lines	↓ proliferation Cell cycle dysregulation ↑ radiation-induced apoptosis	[56, 57]
siRNA	Melanoma and Renal carcinoma cell lines	↑ Apo2L/TRAIL-induced apoptosis	[58]
siRNAs	Endothelial cells	↑ apoptosis ↓ migration on vitronectin ↓ capillary formation	[59]

Table 2 Therapeutic strategies targeted to survivin

Experimental Approach	System	Biological Effects	Ref.
Dominant Negative Mutants			
Cys 84 ' Ala	Melanoma cell lines	↑ spontaneous apoptosis	[40]
Cys 84 ' Ala	Colon cancer cell line	↓ spontaneous apoptosis Cell cycle dysregulation ↓ *in vivo* tumor growth ↓ inhibition of angiogenesis	[46]
Thr 34 ' Ala	Melanoma cell lines	↓ proliferation ↑ spontaneous apoptosis ↑ cisplatin-induced apoptosis ↓ *de novo* tumor formation ↓ tumor growth rate	[47]
Thr 34 ' Ala	Breast, Cervix, Lung and Colorectal cancer cell lines	↓ proliferation ↑ spontaneous apoptosis ↑ taxol-induced apoptosis ↓ de novo tumor formation ↓ tumor growth rate	[48]
CDK Inhibitors			
Flavopiridol	Breast carcinoma and cervical carcinoma cell lines	↑ apoptosis ↑ drug-induced apoptosis ↓ tumor growth rate	[60]
Purvalanol A	Cervical carcinoma cell line	↑ taxol-induced apoptosis ↓ tumor growth rate	[61]

resulting in the cleavage of the RNA target sequences [49]. In particular, the hammerhead ribozyme consists of a highly conserved catalytic core, which cleaves substrate RNA at NHH triplets 3' to the second H, where N is any nucleotide and H is any nucleotide but guanidine. We designed two hammerhead ribozymes targeting the 3' end of the CUA_{110} (RZ7) and the GUC_{294} (RZ1) triplets in the survivin mRNA and transfected them into the JR8 human melanoma cell line overexpressing survivin. Stably transfected clones proven to endogenously express the active ribozyme RZ1 or RZ7 were characterized by a markedly lower survivin protein level than JR8 parental cells, whereas a negligible reduction of survivin expression was observed in cells expressing a mutant ribozyme (which was produced by introducing a mutation in the catalytic core of the active ribozyme RZ1). These cells also demonstrated an increased caspase-9-dependent apoptotic response to cisplatin treatment [50]. JR8 cells expressing RZ1 also showed a significantly increased sensitivity to the topoisomerase-I inhibitor topotecan (as detected by clonogenic cell survival) as a consequence of an enhanced rate of drug-induced apoptosis. Moreover, an increased antitumor activity of oral topotecan was observed in ribozyme-expressing JR8 cells grown

as xenograft tumors in athymic nude mice [51]. JR8 cells endogenously expressing the active RZ7 ribozyme also showed significantly increased sensitivity to γ-irradiation [52]. More recently, we constructed a Moloney-based retroviral vector expressing the RZ7 ribozyme, encoded as a chimeric RNA within adenoviral VA1 RNA. Polyclonal cell populations, obtained by infection with the retroviral vector, of two androgen-independent human prostate cancer cell lines (DU145 and PC-3) were characterized by a significant reduction of survivin expression; the cells became polyploid, underwent caspase-9-dependent apoptosis and showed an altered pattern of gene expression, as detected by oligonucleotide array analysis. Survivin inhibition also increased the susceptibility of these cells to cisplatin-induced apoptosis and prevented tumor formation when cells were xenografted into athymic nude mice [53].

Choi *et al.* [54] also showed that two hammerhead ribozymes, able to cleave the human survivin mRNA at nucleotide position +279 and +28 and cloned into a replication-deficient adenoviral vector, increased the apoptotic response to etoposide in transduced MCF-7 breast cancer cells.

Small interfering RNAs

The discovery that synthetic 21-23 nucleotide RNA duplexes (siRNAs) can trigger an RNA interference response in mammalian cells and induce strong inhibition of specific gene expression has opened the door to the therapeutic use of siRNAs [55]. Specifically, several studies on experimental human tumor models have demonstrated the feasibility of this approach for the inhibition of cancer-related genes including survivin. Carvalho *et al.* [23] firstly used RNA interference to specifically repress survivin in HeLa cells. These authors showed that survivin was no longer detectable in cultures 60h after transfection with specific siRNA and that survivin-depleted cells were delayed in mitosis and accumulated in prometaphase with misaligned chromosomes. Survivin down-regulation, accomplished through the use of siRNAs, was seen to reduce clonogenic potential and increase the percentage of multinucleated cells in a panel of human sarcoma cell lines independently of p53 gene status [56]. Moreover, siRNA-mediated survivin knock down caused radio-sensitization, which was paralleled by an increased activity of caspase-3 and caspase-7, in wt-p53 but not in mutant-p53 sarcoma cells [57]. An enhanced apoptotic response to APO2L/TRAIL treatment was also recently observed in melanoma and renal carcinoma cell lines transfected with survivin-specific siRNAs [58]. Finally, Coma *et al.* [59] recently demonstrated that transefection of endothelial cells with survivin specific siRNAs induced a marked increase in the apoptotic rate, a dose-dependent inhibition of their migration on vitronectin and a decrease in capillary formation.

There is a widely diffused opinion that RNAi provides a powerful tool for targeted inhibition of gene expression, with respect to conventional antisense strategies (*i.e.* ribozymes), presumably because it relies on a natural process. Despite the unique assumed potential of RNAi, limitations in the use of this approach, such as the possibility that some mammalian cells may not be susceptible to RNAi, have been described. Cellular uptake and co localization to the specific target site within cells represent the main hurdles that have to be overcome for an efficient inhibition of gene expression. Although there are still no means to improve co-localization to the target site and to increase the efficacy of siRNAs in the presence of hardly accessible target RNA, a number of specific strategies have been demonstrated to be effective in inducing a sequence-directed co-localization of ribozymes and to improve their efficacy at the target site [60].

Ribozymes as well as siRNAs can lead to non-sequence specific effects (off-target effects) that are strongly dependent on the concentration of oligomers. However, it should be stressed that the double-stranded siRNAs may result in two single-stranded oligomers which yield more pronounced off-target effects than those obtained with an equal molar amount of ribozymes [61]. However, lack of a study aimed to comparatively evaluate the efficacy of ribozymes and siRNAs in inhibiting the expression of the same gene on the same experimental systems makes it difficult to predict which is the better approach to be exploited for therapeutic purposes.

Dominant negative mutants

A different approach to target survivin was developed by Altieri's group using survivin dominant

negative mutants. Grossman *et al.* [43] first demonstrated that transfection of YUSAC2 and LOX melanoma cell lines with a mutant carrying a cysteine 84→alanine (Cys84Ala) mutation in the survivin BIR domain increased the apoptotic index and enhanced the sub-G_1 apoptotic cell fraction in both tumor models. Very recently, Tu *et al.* [62] showed that a gene therapy approach, based on adeno-associated viral vector-mediated transfer of survivin Cys84Ala mutant, induced apoptosis and mitotic catastrophe in colon cancer cells and inhibited angiogenesis and tumor growth in a colon cancer xenograft model *in vivo*.

By using a different dominant negative mutant of survivin characterised by a threonine 34→alanine (Thr34→Ala) mutation that abolishes a phosphorylation site for the cyclin-dependent kinase $p34^{cdc2}$, Grossman *et al.* [63] demonstrated the induction of spontaneous apoptosis in three different melanoma cell systems, YUSAC2, LOX and YUGEN8. As previously reported [16], survivin phosphorylation on threonine 34 is necessary for survivin to associate with processed caspase-9 and to perform its anti-apoptotic function. In addition to inducing apoptosis, transfection of YUSAC2 cells with the Thr34→Ala mutant increased their sensitivity to the anticancer agent cisplatin, as demonstrated by the more than double sub-G_1 apoptotic cell fraction compared to control cells. Conditional expression of Thr34→Ala in YUSAC2 cells prevented tumor formation upon s.c. injection in 13 of 15 CB.17 severely combined immunodeficient (SCID) beige mice. Moreover, such treatment caused a significant reduction (60-70%) in the growth rate of already established tumors and enhancement of apoptosis as revealed by the increased number of TUNEL (terminal deoxynucleotidyltransferase-mediated UTP end labeling)-positive cells [63].

Mesri *et al.* [64] investigated the effect of a replication-deficient adenovirus encoding the Thr34→Ala survivin dominant negative (pAd-T34A) in human tumor cells of various origins *in vitro* and *in vivo*. They showed that infection with pAd-T34A caused spontaneous apoptosis in cell lines of breast, cervical, lung and colorectal cancers, whereas pAd-T34A did not affect the growth of proliferating normal human cells not expressing survivin. In pAd-T34A-infected apoptotic tumor cells the authors observed mitochondrial release of cytochrome *c*, cleavage of caspase-3 and caspase-9, and enhancement of caspase-3 catalytic activity. Moreover, when combined with chemotherapy, pAd-T34A was able to increase the extent of taxol-induced apoptosis, detected as a flow-cytometric sub-G1 apoptotic cell peak, in HeLa and MCF-7 human carcinoma cell lines. *In vivo* experiments on the MCF-7 human breast cancer xenograft model demonstrated that pAd-T34A was able to suppress *de novo* tumor formation, inhibit the growth of already established tumors by approximately 40%, and reduce intraperitoneal tumor dissemination. Moreover, tumors infected with pAd-T34A showed massive apoptosis, as detected by TUNEL staining, and loss of proliferating cells, as demonstrated by low Ki-67 immunoreactivity.

Cyclin-dependent kinase inhibitors

A different strategy focused on pharmacologic inhibition of mitotic phosphrylation of survivin on Thr34 was also pursued to inhibit survivin function.

In this context, inhibitors of cyclin-dependent kinases (CDK) such as flavopiridol or the more $p34^{cdc2}$-specific inhibitor, purvalanol A, were tested in tumor cells arrested at mitosis with taxol, which induces hyperphosphorylation of survivin on Thr34 [36]. Sequential administration of CDK inhibitors resulted in escape from the mitotic block imposed by taxol, marked activation of mitochondrial-dependent apoptosis and anti-cancer activity *in vivo* [37, 65]. These findings provide mechanistic insights into the role of survivin in cytoprotection and suggest that the protein functions in a relatively narrow time window at methaphase that requires elevated $p34^{cdc2}$ activity and polymerized spindle microtubules.

Conclusive remarks

In recent years, studies in which the expression of specific genes was manipulated have provided clues as to how we can intervene to make tumor cells specifically more sensitive to chemical and physical antitumor agents [66]. Such tumor specificity can only be obtained by exploiting a basic difference between normal and malignant cells. In

this context, targeting survivin is particularly attractive because of its high levels of expression in tumor cells and its proven association with disease progression. Overall, the results obtained by different studies aimed at targeting survivin by means of different approaches demonstrated that inhibition of this cytoprotective factor was able to promote spontaneous apoptosis in tumor cells and to enhance the efficacy of several types of conventional treatments including chemotherapy, radiotherapy and immunotherapy. Moreover, the evidence that survivin plays a crucial role also in tumor angiogenesis [67] would suggest that survivin targeting can increase the overall tumor response to treatment not only through direct interference with the apoptotic pathways in cancer cells but also by favouring the apoptotic involution of newly formed tumor vasculature. Clinical testing of survivin antisense oligonucleotides is currently underway.

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