Survivin-3B Potentiates Immune Escape in Cancer but Also Inhibits the Toxicity of Cancer Chemotherapy

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Abstract

Dysregulation in patterns of alternative RNA splicing in cancer cells is emerging as a significant factor in cancer pathophysiology. In this study, we investigated the little known alternative splice isoform survivin-3B (S-3B) that is overexpressed in a tumor-specific manner. Ectopic overexpression of S-3B drove tumorigenesis by facilitating immune escape in a manner associated with resistance to immune cell toxicity. This resistance was mediated by interaction of S-3B with procaspase-8, inhibiting death-inducing signaling complex formation in response to Fas/Fas ligand interaction. We found that S-3B overexpression also mediated resistance to cancer chemotherapy, in this case through interactions with procaspase-6. S-3B binding to procaspase-6 inhibited its activation despite mitochondrial depolarization and caspase-3 activation. When combined with chemotherapy, S-3B targeting in vivo elicited a nearly eradication of tumors. Mechanistic investigations identified a previously unrecognized 7-amino acid region as responsible for the procancerous properties of survivin proteins. Taken together, our results defined S-3B as an important functional actor in tumor formation and treatment resistance.

Introduction

Alternative splicing is an important mechanism for the generation of the variety of proteins indispensable for cell functions. In normal cells, it is finely regulated to orchestrate cellular processes. During cancer development, myriad defects occur throughout cell transformation. Among these defects, alternative splicing misregulation seems to be an important factor. Misregulation of alternative splicing can be observed in cancer cells in the absence of genomic mutations of the genes concerned. In cancer cells, aberrant alternative splicing can give rise to variants that are specifically expressed in cancer tissues and completely absent from normal ones.

Among alternative spliced genes, birc5, or survivin, is important as expression is highly deregulated in cancer. First described in 1997, survivin undergoes alternative splicing that can induce the expression of five different transcripts with different functions: survivin, survivin-ΔEx3, survivin-2B (5), survivin-3B (S-3B; ref. 6), and survivin-2α (7). Although survivin was first described as a tumor-specific gene, over the years it has come to light that it is also expressed in normal cells where its expression is necessary for cell-cycle progression. In the present work, we highlighted that S-3B was a cancer-specific isoform. In addition, overexpression of this variant induced tumorigenesis through the inhibition of tumor-directed immune-cell cytotoxicity by inhibiting death-inducing signaling complex (DISC) formation. Moreover, the expression of S-3B gave a strong advantage to cancer cells exposed to cancer treatment by blocking cell death through the inhibition of procaspase-6 activation. In vivo, the targeting of S-3B in association with chemotherapy, greatly improved tumor response to treatment. Finally, we identified a C-terminal 7-amino acid domain in S-3B as a new protein domain responsible for protein functions.

Materials and Methods

Cell lines

All of the cell lines used (A549, BT474, BT474c, BT483, Calu-3, CCRF-CEM, HBL100, HCC1419, HCC1569, HCC1954, HCC2218, HCT116, HT29, Hs578T, M113, M125, M44, M6, MCF-7, MDA-MB-231, MDA-MB-468, MelC, NCI-H1703, NCI-H1781, NCI-H1975, NCI-H322, NCI-H460, NCI-H520, siHa, SKBr-3, SKOV3, SNB19, SW1116, SW48, SW620, T47D, U373, U87-MG, UACC-812, and Widr) were purchased from American Type Culture Collection (ATCC) or Oncodesign. They were routinely grown in accordance with ATCC-recommended culture conditions. Sensitive and resistant cell lines were purchased from Oncodesign.
Human samples
We studied survivin transcript expression by quantitative real-time PCR (qRT-PCR) in 103 tumors and their adjacent normal tissues from the breast, colon, stomach, kidney, uterus, ovary, and liver. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Centre Georges-François Leclerc (Dijon, France), the Comité Consultatif de Protection des Personnes en Recherche Biomédicale de Bourgogne. Written informed consent was obtained from all patients before enrollment. All samples were treated as described previously (11). The RNA and proteins of 40 carcinomas and their adjacent normal tissues were extracted by TRIzol reagent and protein pellets were kept for Western blotting of the survivin isoform. The same protocol was used for the analysis of tumors generated in mice.

Reverse transcription, semi-quantitative, and quantitative real-time PCR
Reverse transcription and qRT-PCR were conducted as described previously (11, 12). The oligonucleotide sequences used were: S-3B (forward: CCAGATGACGACCCCATAGAG, reverse: CCCCTGACTTCTGTTCAATT, Probe: CATTCTGCCGTGGTGGCTTTCC), mKrb1c (forward: AAGGTTCAATTGGCAGAC, reverse: CACAGCTGTCTTCTAGTG), mEOMS (forward: ACATGCAGGGCAATAAGATG, reverse: GTCACTTGGCCACACTCCTC, reverse: ACTCCGTGAGTTCACCAACC), mPerforin (forward: GTGGGACTTCAGCTTTCCAG, reverse: AGGGTAGATCAT, reverse: GCCCCCAAAGTGACATTTAT), mGranzyme B (forward: ACAAAGGCCACGAGGATCAT, reverse: GCACAGCTGCCATTTTCAGTG), mEOMS (forward: TTAATG-GGCCAACCACCTTC, reverse: ACTCCTGCTGCTTCCACAAACC), and mTRAIL (forward: TGAGTGCCAGAGAACACTCTCA, reverse: TCACCAAGCAGATGAGCG). S-3B overexpression and downexpression
The full-length of the S-3B coding sequence was obtained using SuperScript One-Step long templates RT-PCR (Invitrogen) with 1.25 μg of total RNA. The insert was cloned by using the Vivid Colors pcDNA6.2/CT-YFP-TOPO Mammalian Expression Vector Kit (Invitrogen) as described by the manufacturer.

RNA interference (RNAi) was achieved using S-3B-specific siRNA located in the specific part of the S-3B sequence (forward: AAUUGAGAGAGCUCUGUUAGCGAA, reverse: UUCUGCUACAGACCUUCUCAUU). Scramble RNA with the same nucleotide composition was used as the negative control. The short-hairpin RNA (shRNA) directed against S-3B was obtained (based on siRNA sequence) after DNA duplex insertion into BLOCK-iT Inducible H1 entry Vector (Invitrogen) as described by the manufacturer (validation of specificity in Supplemental Fig. S1B and S1C).

Survivin siRNA was provided by Ambion and its target sequence was located at the junction of exon 3 and 4. This siRNA induced the downregulation of survivin, survivin-ΔEx3, survivin2B, and survivin-2a. S-3B was not influenced by this siRNA.

Western blot analysis
Western blot analyses were conducted as described previously (12) with the following primary antibodies: β-actin, 1:25,000; caspase-8, 1:100; caspase-10, 1:100; Bid, 1:2,000; caspase-9, 1:400; caspase-3, 1:75,000; caspase-6, 1:20,000; caspase-7, 1:1,000; GFP, 1:50,000; Fas-associated protein with death domain (FADD), 1:1,000; Fas, 1:1,000; survivin 1-12, 1:500; survivin, 1:5,000; and β-tubulin, 1:1,000. The S-3B antibody was produced on demand by Millegen by using the KIERALAE peptide as epitope.

Immunoprecipitation assays
Immunoprecipitation assays were conducted on total protein extract with ExactaCruz matrix (Santa Cruz Biotechnology) or Dynabeads (Invitrogen) according to the manufacturer’s instructions and analyzed by Western blot analysis by using the input extract as the Western blot analysis positive control.

Apoptosis, mitochondrial membrane potential assessments by FACS, and crystal violet staining for cytotoxic and clonogenic tests
Cells were treated with Fas ligand (Fasl: 1:20 supernatant), or staurosporine (0.1 μmol/L). Apoptosis was assessed by using the Annexin-V–PE Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer’s instructions.

Mitochondrial membrane potential was assessed after 6 or 24 hours of treatment with Fasl or chemotherapy drugs, respectively, by measuring the reduction of MitoTracker Red CM-H2XRos (Invitrogen) according to the manufacturer’s instructions.

For cytotoxic tests, 2,500 cells were cultured for 72 hours with increasing doses of chemotherapy drugs [staurosporine, 5-fluorouracil (5-FU), epirubicin, etoposide, cisplatin, doxorubicin, vinblastine, and vincristine].

For the natural killer (NK) cell-killing assay, NK cells were obtained from mouse spleen and selected by using CD49b Microbeads (Miltenyi Biotec) and activated for 48 hours with interleukin (IL)-2. Then, MDA-MB-231 cells were seeded and the NK cells were added with an increasing ratio (1 target cell for 200 NK cells). The NK cells were added with an increasing ratio (1 target cell for 200 NK cells).

For clonogenic assays, cells were cultured with staurosporine or docetaxel for 24 hours. The treatment medium was then replaced by classical growth medium and the cells were incubated for 15 days.

At the end of these experiments, the cells still attached to the plastic were stained with 0.5% crystal violet solution. For cytotoxic tests, crystal violet was dissolved in dimethyl sulfoxide (DMSO) and read with a spectrophotometer at 590 nm. For clonogenic assays, colonies containing at least 50 cells were manually counted. All experiments were carried out at least in triplicate.

Determination of X-ray cytotoxicity using clonogenic assay
The radiosensitivity of cell lines was determined by using clonogenic assays according to the method previously described.
The tumors were treated twice a week for 4 weeks. pmol siRNA. Both RNA were injected with Lipofectamine 2000. was applied after one injection of 200
was maintained by new injections once a week throughout the
as the tumor diameter reached 40 mm3, the
experiments were approved by the Ethics Committee of
Federation of European Animal Science Associations. All ani-
characterization of NK cells
At the end of the experiment, MDA-MB-231 tumors were
were separated from tumor cells by Ficoll separation. NK cells were incubated with anti-
CD69, Nkp46, and NK1 antibodies and analyzed by fluores-
cence-activated cell sorting (FACS).
In vivo experiments
All the mice were maintained in specific pathogen-free
conditions and all experiments followed the guidelines of the
Federal Register of European Animal Science Associations. All ani-
mals were approved by the Ethics Committee of Université de Bourgogne (Dijon, France). For the tumorige-
nicity assay, 3 × 106 HBL100 or HBL100-3B cells in serum-free
were inoculated subcutaneously into the right
side of 8-week-old nude (nu/nu) BALB/c mice (Charles River Laboratories; n = 10 with 5 males and 5 females for each set of
cells). Afterward, the tumor diameter was measured weekly using a caliper.
For the S-3B targeting assay, 5 × 106 MDA-MB-231 cells in
were inoculated subcutaneously into the left side of 8-week-old nude (nu/nu) BALB/c mice. Afterward, the tumor diameter was measured weekly using a caliper. As soon as the tumor volume reached 8 or 40 mm3, the
were divided into two groups. The first group (n = 10) received intratumoral injections of 300 pmol scRNA, whereas the second (n = 10) received intratumoral injections of 300 pmol siRNA. Both RNA were injected with Lipofectamine 2000. The tumors were treated twice a week for 4 weeks.
Finally, 5 × 106 Widr or MDA-MB-231 cells in serum-free
culture medium were inoculated subcutaneously into the left side of 8-week-old nude (nu/nu) BALB/c mice. Afterward, the tumor diameter was measured weekly using a caliper. As soon as the tumor diameter reached 40 mm3, the mice were divided into four groups. Two groups (n = 2 × 10) received intratumoral injections of 300 pmol scRNA with 2 injections (at J0 and J8) of either saline or 5-FU into the peritoneum, and the other two groups (n = 2 × 10) received intratumoral injections of 300 pmol siRNA with 2 injections of either saline or 5-FU into the peritoneum. The tumors were treated twice a week for 6 weeks.
Results
S-3B is specifically expressed in carcinomas and promotes tumorigenesis
The protein expression of S-3B was analyzed by Western blot
analysis in 17 cancer cell lines. Its expression was detectable in
16 (94%) cell lines (Fig. 1A and Supplementary Fig. S1A). Then,
its expression was studied in 40 couples of tumors and their
adjacent normal tissues obtained from different organs. What-
ever the organ of origin, S-3B was expressed in the tumor
sample and not in adjacent normal tissues (representative
result in Fig. 1B). To increase the number of patients, we
analyzed, at the transcriptional level, 103 couples of tumors and
their adjacent normal tissues. The data indicated that
the expression of S-3B was significantly tumor specific (Fig. 1C; P < 0.0001).
As S-3B is specifically expressed in tumor tissues and not in
normal tissues, we analyzed the effect of high S-3B expression
on tumorigenesis. For this, we used the nontumorigenic
HBL100 mammary cell line, in which we induced high S-3B
expression (Supplementary Fig. S1B). As controls, we also
analyzed the effect of survivin overexpression and the HBL100
growth in absence of NK cells. After subcutaneous injection of
these cells into nude mice, tumor growth was monitored for 50
days. Although control cells and survivin-overexpressing cells
did not develop into tumors, cells with a high level of S-3B were
able to generate tumors and promote tumor growth (Fig. 1D and
Supplementary Fig. S2A) without increasing cell proliferation in vitro (Supplementary Fig. S2B). Moreover, in absence
of NK cells, the nontumorigenic HBL100 cells were able to
grow (Fig. 2D). In confirmation of these findings, the intratu-
morel injection of an anti-S-3B siRNA caused a considerable
slowdown of tumor growth of highly tumorigenic MDA-MB-
231 cells (Fig. 1E and Supplementary Fig. S2C) once again
without influencing cell proliferation in vitro (Supplementary
Fig. S2D).
S-3B induces resistance of cancer cells to natural killer cell cytotoxicity
As MDA-MB-231 cells are very susceptible to NK cells (16)
and S-3B induced HBL100 cell growth in the presence of NK
cells, we tested the impact of S-3B targeting on tumor growth in
the absence of NK cells. The mice were given intraperitoneal
injections of an NK cell–blocking antibody, and the depletion
of NK cells was maintained throughout the experiment. The
results obtained in the previous experiment were confirmed as
the treatment of tumors with siRNA induced a substantial
slowdown of tumor growth in mice treated with the isotype
antibody (Fig. 2A; CTRL vs. siRNA). The absence of NK cells did
not significantly influence tumor growth of tumors treated
with scramble RNA (Fig. 2A; CTRL vs. Ab). When tumors were
not treated with siRNA in the absence of NK cells, the growth
slowdown induced by siRNA injection was completely lost (Fig.
2A; siRNA vs. siRNA + Ab). Besides, siRNA treatment also had
no effect on tumor growth in the absence of NK cells (Fig. 2A;
Ab vs. siRNA + Ab). These data showed that NK cells were
involved in the slowdown of tumor growth in animals treated
with siRNA. As a comparison, we analyzed the impact of
survivin siRNA injection on MDA-MB-231 tumor growth. The
injection of survivin siRNA decreased tumor growth in the
presence of NK cells but this decrease was slightly less efficient
than S-3B siRNA (Supplementary Fig. S2E).
Then, in nondepleted mice, we assessed the infiltration of
NK cells into tumors. For this, we analyzed the Nkp46
expression by FACS. The data obtained indicated that NK cells were present in both control and siRNA-treated tumors and that the extinction of S-3B did not influence the infiltration of NK cells (Fig. 2B). This result suggested that tumor behavior in response to siRNA was not dependent on the infiltration of NK cells into the tumor. Once infiltrated, NK cells are activated. We analyzed the transcriptional expression of mouse Granzyme B, EOMS, Perforin, FasL, and Trail in control tumors and siRNA-treated tumors. We also analyzed the membrane expression of CD69 and NK1 by flow cytometry. As shown in Fig. 2C, the activation level of NK cells was the same whatever the tumor treatment (Fig. 2C). In Fig. 2D, the characterization of NK cells indicated that Nkp46+/CD69+ and NK1+/CD69+ cells were present in the same proportion in tumors. Finally, we conducted a killing assay by using IL-2–activated NK cells obtained from mice or FasL. In S-3B–overexpressing MDA-MB-231 cells, NK cells and FasL had a very slight cytotoxic effect compared with control cells. In contrast, the complete absence of S-3B strongly sensitized MDA-MB-231 cells to NK cell and FasL cytotoxicity (Fig. 2E and F).

Taken together, these data showed that S-3B made cancer cells resistant to the cytotoxic activity of NK cells.

**S-3B inhibits cytotoxic cell–induced apoptosis through the inhibition of DISC assembly**

NK cells induce cell death through two different pathways: death domain receptors (Fas/FasL, Trail) and Granzyme B. Although the death domain receptor pathway is the predominant pathway for tumor cell killing by NK cells, especially Fas/FasL (17), Granzyme B did not seem to be necessary for NK cell–mediated tumor rejection (18). We therefore explored apoptosis induced by NK cells through the activation of the death domain receptor Fas. Apoptosis induced by FasL was inhibited by S-3B overexpression and strongly amplified in cells treated with si/shRNA without influencing the expression of Fas (Fig. 3A). To the contrary, the survivin expression variation had no effect on cells sensitivity (Supplementary Fig. S3A). Moreover, the analysis of mitochondrial depolarization showed that the inhibition occurred upstream from the mitochondria (Fig. 3B).
Western blot analysis experiments indicated that S-3B was able to block the induction of extrinsic apoptosis (inhibition of activation of caspase-8, caspase-10, inhibition of cleavage of Bid in tBid, and inhibition of caspase-3 activation) when it was overexpressed, whereas its downregulation augmented the effect of FasL treatment on these proapoptotic proteins (Fig. 3C and Supplementary Fig. S3B). As the interaction between Fas and FasL induces the formation of DISC, we analyzed the involvement of S-3B in the formation of DISC. Using immunoprecipitation experiments, we highlighted that S-3B was able to interact with procaspase-8 (Fig. 3D) but not with procaspase-10 (Supplementary Fig. S3C). This interaction induced the sequestration of procaspase-8 and the inhibition of any interaction between procaspase-8 and FADD (Fig. 3D). These results indicated that S-3B inhibited the DISC by inhibiting extrinsic pathways.

S-3B inhibits cancer treatment-induced cell death

As shown above, S-3B is able to inhibit extrinsic apoptosis by directly interacting with procaspase-8 and thus blocking DISC formation. As S-3B has already shown its ability to inhibit
extrinsic apoptosis, we tested whether S-3B could play a role in the intrinsic apoptosis pathway triggered by Granzyme B (19) or cancer treatments. We used staurosporine, which is known to induce intrinsic apoptosis. Although the overexpression of S-3B greatly reduced the cytotoxicity of staurosporine, its extinction sensitized cells to the drug (Fig. 4A). Using FACS with Annexin V/7-AAD staining experiments, we confirmed that cell death induced by staurosporine was apoptosis, and that, contrary to survivin (Supplementary Fig. S4A), S-3B protected cells from this stress (Fig. 4B and Supplementary Fig. S4B). Moreover, S-3B had a real protective effect as cells overexpressing S-3B had a strong clonogenic capacity after staurosporine treatment (Supplementary Fig. S4C).

We next tested the influence of S-3B on the mitochondrial depolarization that occurred during treatment with staurosporine. Surprisingly, it seemed that the inhibition of cell death by S-3B occurred downstream from the mitochondria (Fig. 4C). At the protein level, treatment with staurosporine was accompanied by the activation of caspase-9, -3, and -7 in control, S-3B–overexpressing, and S-3B–downexpressing cells. However, it seemed that the activation of procaspase-6 was influenced by S-3B. Although the overexpression of S-3B reduced the cleavage of procaspase-6, the absence of S-3B (siRNA in Fig. 4D).

Figure 3. S-3B inhibits cytotoxic cell induced apoptosis through the inhibition of DISC assembly. A, apoptosis assay was conducted by FACS by using Annexin V/7-AAD staining after FasL treatment. Compared with MDA-MB-231 control cells (CTRL), S-3B–overexpressing cells (S-3B) were less sensitive to FasL exposure, whereas S-3B–downexpressing cells (si/shRNA) showed strong sensitivity to FasL. Moreover, the expression variation of S-3B did not influence Fas protein expression in cells. B, mitochondrial depolarization analysis showed that, unlike control cells, S-3B–expressing cells did not show depolarization of mitochondria, whereas about 90% of cells without S-3B (si/shRNA) presented mitochondrial depolarization (n = 3). C, the activation of caspases-8, -10, and -3 and the cleavage of Bid in tBid in response to FasL were analyzed by Western blotting. The overexpression of S-3B inhibited the activation of caspases-8, -10, and -3, and the appearance of Bid. The extinction of S-3B (si/shRNA) dramatically increased the activation of these proteins. Band density was assessed by using ImageJ software and β-actin was used for loading normalization. Bar graphs represent the mean ± SD of three different experiments. D, immunoprecipitation experiments after 24-hour FasL exposure showed that S-3B interacted with procaspase-8 to inhibit DISC formation. Thus, procaspase-8 was unable to interact with FADD and therefore could not be activated by proteolytic cleavage. All experiments were repeated at least three times. *** P < 0.001. IP, immunoprecipitation.
transfection or shRNA expression) accentuated the disappearance of the caspase-6 zymogene (Fig. 4D and Supplementary Fig. S5A). This observation was extremely important as it showed that the depolarization of mitochondria and the activation of caspases-9 and -3, which are normally synonymous with apoptosis, were not followed by cell death in the presence of either 5-FU (25 mg/kg) or saline. Top, in mice injected with saline, tumors treated with scramble RNA (CTRL), like the tumors treated siRNA, showed a high level of apoptosis. C, mitochondrial depolarization analysis showed that, in response to staurosporine, S-3B did not influence mitochondrial depolarization whatever its expression level. D, the activation of caspases-9, -3, -6, and -7 in response to staurosporine was analyzed by Western blotting. The overexpression of S-3B (S-3B) inhibited the activation of caspase-6 without inhibiting caspases-9, -3, and -7. The extinction of S-3B (si/shRNA) dramatically increased the cleavage of procaspase-6. Caspase-9, -3, and -7 activation was not influenced by the downregulation of S-3B in response to staurosporine. E, immunoprecipitation experiments after 48 hours of treatment with staurosporine showed that S-3B interacted with procaspase-6 to inhibit its association with caspase-3 and therefore its activation. Thus, S-3B did not interact with caspase-3. F, Widr (top) and MDA-MB-231 (bottom) cells were subcutaneously injected into nude mice. As soon as tumor volumes reached 40 mm³ (day 0), anti-S-3B siRNA (or scramble RNA as control) was injected into the tumor every 4 days for 40 days. The mice were given two intraperitoneal injections (J0 and J8) of either 5-FU (25 mg/kg) or saline. Top, in mice injected with saline, tumors treated with scramble RNA (CTRL), like the tumors treated siRNA (siRNA), presented exponential growth due to resistance of Widr cells to NK-cell toxicity. In mice injected with 5-FU, for the tumors injected with scramble RNA, the chemotherapeutic treatment induced a slight delay in tumor growth but not a decrease in tumor size. The combination 5-FU treatment + siRNA injection (5-FU + siRNA) induced a strong continuous stabilization of tumor volume throughout the monitored time. Bottom, in mice injected with 5-FU, for the tumors injected with scramble RNA, the chemotherapeutic treatment induced a delay in tumor growth but not a decrease in tumor size. The combination 5-FU treatment + siRNA injection (5-FU + siRNA) induced a stabilization followed by a slight decrease of tumor volume throughout the monitored time. Tumor sizes are plotted as mean ± SD for 10 mice/group. ***; P < 0.001. IP, immunoprecipitation.

Figure 4. S-3B inhibits cancer treatment-induced cell death. A, cytotoxic tests were carried out on MDA-MB-231 cells overexpressing or downexpressing (stably or transiently) S-3B. Cells were exposed to increasing doses of staurosporine (μmol/L) for 48 hours. The percentage of living cells is plotted as mean ± SD (n = 3–6). Compared with control cells (CTRL), S-3B-overexpressing cells (S-3B) were less sensitive to staurosporine, whereas S-3B-downexpressing cells (si/shRNA) showed strong sensitivity to this drug. B, the apoptosis assay was conducted by FACS by using Annexin V/7-AAD staining after staurosporine treatment. These data showed that, compared with control cells (CTRL), overexpressing cells (S-3B) were more resistant to apoptosis, whereas S-3B-downexpressing cells (si/shRNA) showed a high level of apoptosis. C, mitochondrial depolarization analysis showed that, in response to staurosporine, S-3B did not influence mitochondrial depolarization whatever its expression level. D, the activation of caspases-9, -3, -6, and -7 in response to staurosporine was analyzed by Western blotting. The overexpression of S-3B (S-3B) inhibited the activation of caspase-6 without inhibiting caspases-9, -3, and -7. The extinction of S-3B (si/shRNA) dramatically increased the cleavage of procaspase-6. Caspase-9, -3, and -7 activation was not influenced by the downregulation of S-3B in response to staurosporine. E, immunoprecipitation experiments after 48 hours of treatment with staurosporine showed that S-3B interacted with procaspase-6 to inhibit its association with caspase-3 and therefore its activation. Thus, S-3B did not interact with caspase-3. F, Widr (top) and MDA-MB-231 (bottom) cells were subcutaneously injected into nude mice. As soon as tumor volumes reached 40 mm³ (day 0), anti-S-3B siRNA (or scramble RNA as control) was injected into the tumor every 4 days for 40 days. The mice were given two intraperitoneal injections (J0 and J8) of either 5-FU (25 mg/kg) or saline. Top, in mice injected with saline, tumors treated with scramble RNA (CTRL), like the tumors treated siRNA (siRNA), presented exponential growth due to resistance of Widr cells to NK-cell toxicity. In mice injected with 5-FU, for the tumors injected with scramble RNA, the chemotherapeutic treatment induced a slight delay in tumor growth but not a decrease in tumor size. The combination 5-FU treatment + siRNA injection (5-FU + siRNA) induced a strong continuous stabilization of tumor volume throughout the monitored time. Bottom, in mice injected with 5-FU, for the tumors injected with scramble RNA, the chemotherapeutic treatment induced a delay in tumor growth but not a decrease in tumor size. The combination 5-FU treatment + siRNA injection (5-FU + siRNA) induced a stabilization followed by a slight decrease of tumor volume throughout the monitored time. Tumor sizes are plotted as mean ± SD for 10 mice/group. ***; P < 0.001. IP, immunoprecipitation.
of S-3B, as shown by apoptosis and clonogenic assays (Fig. 4C and Supplementary Fig. S4B).

As S-3B inhibited procaspase-6 activation without inhibiting caspase-3 activity in response to staurosporine, we analyzed the putative interaction between S-3B and procaspase-6. Using immunoprecipitation experiments, we showed that S-3B interacted with procaspase-6 and inhibited its interaction with active caspase-3 (Fig. 4E). These observations showed that S-3B blocked procaspase-6 activation, and therefore the cleavage of its substrates, and finally apoptosis. We also analyzed whether S-3B was also able to interact with survivin. After staurosporine treatment, no interaction between S-3B and survivin was observed (Supplementary Fig. S5B). The inhibition of apoptosis by S-3B was also present in response to 5-FU (by the same mechanism described above; Supplementary Fig. S5C), epirubicin, etoposide, cisplatin, X-rays, docetaxel, and vincristine (Supplementary Fig. S6).

As S-3B played an important role in the protection of cells against apoptotic stimuli, we analyzed the expression of S-3B in six cancer cell lines (A549, HL60, K562, KBV1, MCF-7, and RPMI) that were sensitive or resistant to docetaxel, adriamycin, doxorubicin, or vinblastine. The data obtained indicated that S-3B expression increased with the acquisition of resistance (Supplementary Fig. S7). As the involvement of S-3B in cancer cell resistance was confirmed, we tested the impact of S-3B targeting in vitro on the behavior of different cancer cell lines from different tissues (breast, brain, colon, and cervix) in response to different drugs. S-3B targeting sensitized all tested cell lines to numerous tested drugs (Supplementary Table S1), and notably to drugs that are used in clinics as the reference treatment (5-FU and docetaxel for breast cancer, 5-FU and cisplatin for colon cancer, vincristine and vinblastine for brain cancer, and cisplatin and doxoroxel for cervical cancer).

Finally, we carried out in vivo experiments to explore the therapeutic effect of targeting S-3B. For this, we used colon cancer NK-resistant Widr cells (20) and the mammary cancer NK-sensitive MDA-MB-231 cells. By using these cell lines, it was possible to observe the impact of chemotherapy and S-3B targeting alone, in the absence of NK-cell toxicity, on tumor growth for Widr cells. With MDA-MB-231 cells, we could observe a possible synergistic effect between NK cell toxicity and chemotherapy treatment. We combined intratumoral injections of siRNA with systemic 5-FU. As expected in Widr cells (Fig. 4F, top), the intratumoral injection of siRNA did not affect tumor growth even in the presence of NK cells (CTRL vs. siRNA lines). Treatment with 5-FU slightly decreased tumor growth compared with control (CTRL vs. 5-FU lines). The combination of treatment with 5-FU and injections of siRNA induced a stabilization of tumor volume, showing that siRNA had a significant positive effect on tumor sensitivity to chemotherapy (5-FU vs. 5-FU+siRNA lines). For MDA-MB-231 cells (Fig. 4F, bottom), 5-FU treatment decreased tumor growth compared with control (CTRL vs. 5-FU lines). The combination of siRNA and 5-FU resulted in a stabilization followed by a decrease of tumor volume (5-FU+siRNA line). These data clearly showed that S-3B could be a promising therapeutic target.

The C-terminal domain of S-3B is indispensable for its protective properties

S-3B possesses a complete BIR domain and a specific 7-amino acid sequence (that we named the LEO domain) at the C-terminal: ERALLAE (Fig. 5A). For these experiments, we generated cells that stably overexpressed truncated S-3B (S-3B without the LEO domain). The truncated S-3B was unable to protect cells against staurosporine (Fig. 5B and Supplementary Fig. S8). At the protein level, unlike overexpression of the full S-3B, overexpression of the truncated form was unable to inhibit the activation of procaspase-6 (Fig. 5C). This absence of inhibition was explained by the lack of any interaction between truncated S-3B and procaspase-6 (Fig. 5D). For extrinsic apoptosis, the same observations were made. Truncated S-3B was unable to inhibit procaspase-8 activation (Fig. 5E and Supplementary Fig. S9) due to the deficiency of any interaction between truncated S-3B and procaspase-8 (Fig. 5F). The importance of the LEO domain was confirmed by cytotoxic tests in response to X-rays and clonogenic tests in response to staurosporine (Supplementary Fig. S10A and S10B, respectively). Taken together, these results showed that the protection properties of S-3B involve an unsuspected new protein domain that comprises 7 amino acids (LEO domain).

Discussion

The deregulation of alternative splicing in cancer can be responsible for the tumor-specific expression of protein. The appearance of these proteins gives strong advantages to malignant cells for their development and propagation throughout the organism. Moreover, their tumor-specific expression profile makes them potential therapeutic targets. Here, we showed that the alternative splice variant S-3B is specifically expressed in tumors from various organs (breast, colon, kidney, etc.), and that its expression can play a role in tumorigenesis. Indeed, its overexpression allowed the growth of nontumorigenic cells in nude mice. We also showed that this tumorigenesis capacity was due to the resistance of cancer cells to the toxicity of NK cells. We pointed out that this inhibition was due to a specific interaction of S-3B with procaspase-8. This interaction completely blocked the formation of DISC, which is the first step of the extrinsic apoptosis pathway (21). S-3B, like FLIP, is a new inhibitor of the DISC (22). Consequently, S-3B allowed the immune escape of cells, as tumor cell growth was diminished by NK cells when S-3B was downregulated. The immune escape of malignant cells is directly involved in cancer development, whatever the tissue of origin, and is now a potential target for clinical investigations on the microenvironment and NK cells (23). In addition, the targeting of S-3B could be a potential means to sensitize tumor cells to the immune system without the risk of modifying exogenous NK cells. Indeed, it seemed that the infiltrating NK cells were identically activated and in same proportion in siRNA-treated tumors compared with control ones.

Another important aspect of the impact of S-3B on cancer cells is its ability to confer resistance to anticancer treatments. Up to now, only two studies have reported S-3B as a potential antiapoptotic protein in response to cisplatin (24) or 5-FU (25).
In our work, we showed that S-3B expression dramatically increased during the acquisition of resistance to chemotherapy. It is now accepted that mitochondrial depolarization is a point-of-no-return of the lethal process (26) and that the presence of active caspase-3 is a marker of apoptosis (27), and therefore cell death. In this study, we showed that in response to activation of the intrinsic apoptosis pathway, S-3B acted downstream from the mitochondria by preventing active caspase-3-mediated cleavage of procaspase-6 (at least after staurosporine and 5-FU treatment). Despite mitochondrial depolarization and caspase-3 activation, cells that overexpressed S-3B did not die as indicated by their high clonogenic potential. Furthermore, a recent study described the important role of active caspase-3 in cancer. Caspase-3 activation during the apoptotic process stimulates tumor cell repopulation after radiotherapy (28). On the basis of this work, we can imagine that, in the presence of a high level of S-3B, the activation of caspase-3 in response to radio- or chemotherapy could potentiate the metastatic capacity of tumor cells. As cells did not die, this could amplify the metastasis phenomenon rather than the

Figure 5. The C-terminal domain of S-3B is indispensable for its protective properties. A, the three-dimensional structure showed that S-3B possesses a BIR domain and harbored a specific 7-amino acid domain ERALLAE. B, cytotoxic tests were carried out on MDA-MB-231 cells overexpressing (stably or transiently) YFP (CTRL), S-3B, or S-3B truncated for its specific 7-amino acid domain. Living cells were stained with crystal violet. The percentage of living cells is plotted as mean ± SD (n = 3–6). S-3B–overexpressing cells (S-3B) were more resistant than control cells (CTRL). In contrast, cells with truncated S-3B showed the same sensitivity as control cells. C, the activation of caspase-6 in response to staurosporine was analyzed by Western blot analysis. After treatment with staurosporine, the overexpression of S-3B (S-3B) inhibited the activation of caspase-6. The truncation of S-3B (truncated S-3B) induced the loss of this inhibitory potential. Band density was assessed by using ImageJ software and β-actin was used for loading normalization. Bar graphs represent the mean ± SD of three different experiments. D, immunoprecipitation after 48 hours of treatment with staurosporine indicated that S-3B interacted with procaspase-6, whereas truncated S-3B was unable to bind procaspase-6. E, the activation of caspases-8 in response to FasL was analyzed by Western blot analysis. The overexpression of S-3B (S-3B) inhibited the activation of caspases-8. As in control cells (CTRL), the truncation of S-3B (truncated S-3B) did not inhibit the activation of these proteins. Band density was assessed by using ImageJ software and β-actin was used for loading normalization. Bar graphs represent the mean ± SD of three different experiments. F, immunoprecipitation after 48 hours of treatment with staurosporine indicated that S-3B interacted with procaspase-8, whereas truncated S-3B was unable to bind this procaspase. All experiments were repeated at least three times. *** P < 0.001; #, nonsignificant. IP, immunoprecipitation.
repopulation of the tumor site. This may explain the previously reported poor disease-free and overall survival in patients with breast carcinoma expressing a high level of S-3B and treated with neoadjuvant chemotherapy (29, 30). The roles of S-3B in the inhibition of apoptosis are summarized in Fig. 6.

We showed that S-3B could be a therapeutic target in association with chemotherapy. The extinction of S-3B by siRNA combined with treatment with 5-FU clearly decreased tumor volume. These results clearly indicated that targeting S-3B sensitized tumor cells to chemotherapy. Currently, the survivin suppressor YM155 is under clinical development in 3B sensitized tumor cells to chemotherapy. Currently, the survivin suppressor YM155 is under clinical development in phase I/II studies. It has proved to be quite well tolerated but has relatively low efficacy (31, 32). Nevertheless, this small molecule might not be working by survivin suppression alone, which could explain its low toxicity and limited efficacy (33). Contrary to survivin, whose expression is necessary for mitotic progression in normal cells (10), S-3B is only present in tumors, making S-3B a very interesting therapeutic target with potentially low levels of side effects. This suggests that the systemic targeting of S-3B could be considered a potentially safe and effective therapy for clinical trials.

Finally, unlike other BIR domains, which contain proteins that interact with active caspases-3 and -7, but not with caspase-8 (34), S-3B is able to bind procaspase-8 and procaspase-6. Here, we showed that S-3B interacted with procaspase-8 and procaspase-6 thanks to its 7-amino acid C-terminal domain, which we named the LEO domain.

In conclusion, this work (i) identified S-3B as an actor of tumor immune escape and tumor resistance to cancer treatment; (ii) highlighted the fact that despite mitochondrial depolarization and caspase-3 activation, S-3B expressing cells did not die; (iii) pointed out that S-3B could be a good therapeutic target as targeting S-3B decreased tumor volume, and as S-3B expression is tumor specific, suggesting the potential selectivity of malignant tissues; (iv) identified a new and unsuspected protein domain (the LEO domain) as a potential new protein domain with cytoprotective properties.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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