Peptides and Aptamers Targeting HSP70: A Novel Approach for Anticancer Chemotherapy

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Abstract

The inhibition of heat shock protein 70 (HSP70) is an emerging strategy in cancer therapy. Unfortunately, no specific inhibitors are clinically available. By yeast two-hybrid screening, we have identified multiple peptide aptamers that bind HSP70. When expressed in human tumor cells, two among these peptide aptamers—A8 and A17—which bind to the peptide-binding and the ATP-binding domains of HSP70, respectively, specifically inhibited the chaperone activity, thereby increasing the cells’ sensitivity to apoptosis induced by anticancer drugs. The 13-amino acid peptide from the variable region of A17 (called P17) retained the ability to specifically inhibit HSP70 and induced the regression of subcutaneous tumors in vivo after local or systemic injection. This antitumor effect was associated with an important recruitment of macrophages and T lymphocytes into the tumor bed. Altogether, these data indicate that peptide aptamers or peptides that target HSP70 may be considered as novel lead compounds for cancer therapy.

Introduction

Stress-inducible heat shock protein 70 (HSP70) is a prominent cytoprotective factor. Under normal conditions, HSP70 functions as an ATP-dependent chaperone by assisting the folding of newly synthesized proteins and polypeptides, the assembly of multiprotein complexes, and the transport of proteins across cellular membranes (1–3). HSP70 upregulation by cellular stress or transfection-enforced HSP70 overexpression inhibits apoptosis induced by a wide range of insults and may facilitate oncogenic transformation (4, 5). Thus, HSP70 overexpression increases the tumorigenicity of cancer cells in rodent models (6) and correlates with poor prognosis in patients (7). Conversely, HSP70 downregulation is sufficient to kill tumor cells or to sensitize them to apoptosis induction in vitro (8) and can reduce tumorigenicity in vivo (9). The antia apoptotic function of HSP70 involves interactions with several components of the apoptotic machinery. HSP70 has been demonstrated to bind to Apaf-1, thereby preventing the recruitment of procaspase-9 to the apoptosome (10). Moreover, HSP70 can inhibit apoptosis by directly neutralizing the caspase-independent death effector, apoptosis inducing factor (AIF; 11).

Targeting HSP70 is an emerging concept in cancer therapy. Different inhibitors of HSP90 are being tested in clinical trials. These are mainly compounds derived from the geldanamycin antibiotic, such as the 17-allylamino-17-demethoxygeldanamycin (17AAG), but they also include synthetic small molecules designed to bind to the ATP domain of HSP90 (12). Like the synthetic molecules, geldanamycin derivatives also associate with the HSP90 ATP domain, thus inhibiting ATP binding and, therefore, affecting the function of signaling proteins whose structure depends on the HSP90 chaperone activity (13, 14). Currently, 17AAG is being tested for its chemosensitizing effects in phase III clinical trials, with encouraging results in multiple myeloma (15).

HSP70 can be targeted by a "negative" strategy, that is, siRNAs or antisense oligonucleotides to downregulate its expression (8, 9). In addition, we have shown the feasibility of a "positive" HSP70-targeting, chemosensitizing strategy in which a molecule that antagonizes HSP70 at the protein level is introduced into cancer cells. Based on our previous results, which showed that HSP70 specifically binds to AIF and sequesters it in the cytosol (16), we designed a construct, encoding the minimal AIF region required for HSP70 binding. This AIF derivative, called ADD70 (for AIF-Derived Decoy for HSP70), interacts with the peptide-binding domain of HSP70, thereby inhibiting the interaction of HSP70 with AIF and other client proteins. ADD70 was not cytotoxic on its own, yet it displayed chemosensitizing properties in vitro and in vivo in rodent models (17). Confirming the interest in neutralizing HSP70 in cancer therapy, 2-phenylethynesulfonamide (PES), a

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recently introduced small inhibitor of HSP70, has been described to retard tumor growth in a mouse model of MYC-driven lymphoma (18, 19).

Taking into account the antiapoptotic and oncogenic functions of HSP70 and the fact that very few molecules specifically target HSP70, we sought to construct small peptides that target additional molecular surfaces of HSP70, which may serve as lead compounds for the development of small HSP70 inhibitors. We report here the mechanistic exploration of the anticancer effects of HSP70-targeting aptamers and provide a proof-of-principle that such peptides can inhibit tumor growth in vivo.

### Materials and Methods

#### Cells, plasmids, transfections, and products

HeLa cells [provided by American Type Culture Collection (ATCC), 2007]. Mouse embryonic fibroblasts (MEF; ATCC 2007), HSF1−/− MEF (heat shock factor 1−/−; 20), and HSP70.3−/− HSP70.3−/− MEFs (MEF HSP70−/−; 21) are cultivated in DMEM 10% FBS (Lonza); the mouse B16F10 melanoma cell line (ATCC) is cultivated in RPMI 10% FBS (Lonza); and the rat colon cancer PROb cells (22) are cultivated in HAM’S F10 10% FBS (Lonza). Transfections were done by using the Superfect reagent (Qiagen) or the Chariot transfection apparatus (Bio-Rad). After blocking nonspecific binding with 5% (w/v) nonfat dry milk, membranes were first probed overnight using primary antibodies: HA-tag antibody was from Cell Signaling (Ozyme; ref. 23). Next, the membranes were incubated for 1 hour with appropriate secondary antibodies coupled to horseradish peroxidase (Jackson ImmunoResearch laboratories) and revealed with ECL (Amersham).

#### Immunoprecipitation and Western blotting

Transfected cell (HSP70, aptamers) were lysed in lysis buffer [50 mmol/L HEPES (pH 7.6), 150 mmol/L NaCl, 5 mmol/L EDTA, and 0.1% NP40], incubated with HA-tag antibody (16B12 clone), and subjected to immunoblotting. For the in vitro communoprecipitation, we used HSP70 WT or the corresponding mutants (both HA-tagged) with purified peptides (biotin-tagged) or peptide aptamers (MYC-tagged). The peptide aptamers were produced with the TNT Quick Coupled Transcription/Translation System as follows: 1 μg of template plasmid DNA was added to the reaction mixture that was later incubated at 30°C for 90 minutes. Immunoprecipitates were separated in a 10% or 14% SDS-polyacrylamide gel and transferred to nitrocellulose membranes using a wet transfer apparatus (Bio-Rad). After blocking nonspecific binding with 5% (w/v) nonfat dry milk, membranes were first probed overnight using primary antibodies; HA-tag antibody was from Covance (Eurowgentec); HSC70 (B-6 clone) was from Santa-Cruz (TebuBio); and the MYC-tag antibody (9B11 clone) was from Cell signaling (Ozyme; ref. 23). Next, the membranes were incubated for 1 hour with appropriate secondary antibodies coupled to horseradish peroxidase (Jackson ImmunoResearch laboratories) and revealed with ECL (Amersham).

#### Cell death analysis

The 2.5 × 10⁵ adherent plates were plated onto 6-well culture plates in a complete medium. When indicated, cells were treated with cisplatin (CDDP, 12.5, 25, or 50 μmol/L), etoposide, or 5-fluorouracil (5-FU; 10 μmol/L) for 24 hours, and/or the same was applied to peptides P0, P8, and P17 (1−5 mg/L, 24 hours). Cell death was measured by the crystal violet colorimetric assay or Hoechst 33342 (Sigma-Aldrich) staining. For PS exposure, 10⁵ cells stained with propidium iodide (PI) and FITC-Annexin V conjugate were analyzed by flow cytometry with a FACS Scan flow cytometer (Becton Dickinson). Caspase-3 activity was determined by using the fluorochrome FITC-DEVD-fmk (PromoKine Caspase-3 staining kit; PromoCell).

#### HSP70 chaperone activity

HSP70 chaperone activity was evaluated with a protein thermolability assay. Recombinant HSP70, HSP90, or HSC70 were added (3.5 kg/mL; Stressgen, TebuBio), with or without the molecules to test (100 kg/mL), to 2 mg/mL of total proteins from HSF1−/− MEFs (Dc Assay kits; Bio-Rad). The mixture was heated at 55°C for 1 hour. After centrifugation to eliminate the aggregated proteins, the remaining native proteins in the supernatant were quantified. The ratio between the initial amount of soluble proteins and that obtained after heating allowed for the quantification of protein aggregation.

#### Tumor growth analysis in vivo

Exponentially growing B16F10 cells (wild-type and aptamer-transfected) were harvested and resuspended in an RPMI medium without FBS to a concentration of 2 × 10⁶/mL. B16F10 cells. In vivo studies were performed in wild-type or nu/nu C57/BL6 mice (Charles River). B16F10 cells (5 × 10⁶ cells) were injected s.c. into the right flank. Tumor volumes were measured every 2 days. The animals were treated according to the guidelines of the Ministère de la Recherche et de la Technologie, France. All experiments were approved by the Comité d’Éthique de l’Université de Bourgogne.

#### Histologic study of the tumor

Animals were killed 14 or 19 days after cell injection. The site of the tumor cell injection was resected and snap-frozen in methylbutane that had been cooled in liquid nitrogen. An immunohistochemical study of tumor-infiltrating inflammatory cells was done on acetone-fixed 5-μm cryostat sections. Two independent experiments were done in which 4 mice were injected with the different cells.

#### Isolation of plasma lipoproteins by gel filtration and analysis of the peptides by MALDI-TOF

Total lipoproteins were isolated from human plasma in the d < 1.21 g/mL fraction and were dialyzed overnight against PBS. For each aptamer, mixtures of lipoproteins (1.9 mg/mL of protein) and peptide (0.7 mg/mL) were incubated for 1 hour
prior to being fractionated by gel permeation chromatography on a Superose 6HR column. Peak fractions containing individual lipoproteins (VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL: high density lipoproteins) were delipidated with 100 volumes of ethanol to diethyl ether (3:2). The delipidated lipoprotein fraction was then mixed with 9 volumes of α-cyano-hydroxy-cinnamic acid (1 mg/mL) dissolved in a ratio of acetonitrile to trifluoroacetic acid to H2O (60:0.1:30, v:v:v). Peptides were spotted on a ground steel plate and analyzed by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry on an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonique S.A.) in the reflectron mode.

**Statistical methods**

For in vitro experiments, Student’s t test and the ANOVA (mean ± SD) were used for statistical analysis as appropriate. All P values were calculated using 2-sided tests, and error bars in the graphs represent 95% CIs. For the analysis of HSP70 activity, we used a repeated-measure ANOVA model and evaluated with Holm-Sidak.

**Results and Discussion**

**Selection of HSP70-binding peptide aptamers**

An optimized yeast 2-hybrid procedure was used to select peptide aptamers for their ability to bind to HSP70 (24). Two peptide aptamers libraries, consisting of an *Escherichia coli* thioredoxin scaffold displaying variable peptide loops of 8 or 13 amino acids and both of a complexity of 2.5 × 10^11 transformants (25), were screened. We selected 8 aptamers with a variable region of 8 amino acids and 9 aptamers with a variable region of 13 residues (Table 1). To find the capacity of these aptamers to bind to endogenous HSP70 in mammalian cancer cells, we cloned the aptamer coding sequences into an HA-tagged pcDNA3 vector and transiently transfected them into HeLa cells. We then immunoprecipitated the aptamers with an HA-tag antibody (Fig. 1A, bottom blots) and revealed the endogenous HSP70 bound by immunoblot (Fig. 1A, top blot). HSP70 was coimmunoprecipitated to various extents with most peptide aptamers. Four peptide aptamers (A8, A11, A12, and A17) exhibited particularly strong binding to HSP70 (Fig. 1A).

HSP70 inactivation in cancer cells sensitizes them to apoptotic killing by anticancer chemotherapeutics (9, 21, 26). Therefore, we analyzed the chemosensitizing properties of the 17 selected aptamers. HeLa cells transiently transfected with aptamer expression vectors were treated with the anticancer agent cisplatin, and cell survival was assessed. None of the aptamers exhibited any cytotoxicity on their own (Fig. 1B). However, 2 aptamers (A8 and A17) strongly sensitized the cells to killing by cisplatin (Fig. 1B). Importantly, A8 and A17 belonged to the group of peptide aptamers that showed the highest apparent binding affinity for HSP70 (Fig. 1A), inciting us to continue their characterization.

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HeLa cells were mock-transfected or transiently transfected with expression vectors that either coded for an HA-tagged control aptamer, which did not bind to HSP70 in the yeast 2-hybrid assay (A0), or for the HSP70-targeted aptamers A8 or A17. Then, the cells were treated with different concentrations of cisplatin, and cell death was determined by a crystal violet colorimetric assay. As shown in Figure 2A, none of the aptamers induced cell death on its own. However, after cisplatin treatment, the aptamers A8 and A17 increased the percentage of cell death, for example, by a factor of 3 to 4 for a concentration of cisplatin of 25 μmol/L during 24 hours. That this cell death was apoptosis was determined by counting the cells presenting chromatin condensation (Hoechst 33342), PS exposure (FITC-Annexin V), and caspase-3 activation (FITC-DEVD-fmk; Fig. 2B–D). Figure 2E shows that the sensitizing effect of the HSP70 peptide aptamers was not just specific for cisplatin but was a more general effect since A8 and A17 also strongly increased apoptosis induced by other chemotherapeutic drugs such as 5-FU or etoposide. A similar sensitizing effect to apoptosis was obtained in mouse melanoma B16F10 cells that were stably transfected with A8 or A17 (Supplementary Fig. 1).

**Table 1. The amino-acid sequences of the variable regions of the selected peptide aptamers**

<table>
<thead>
<tr>
<th>Peptide aptamers</th>
<th>Sequence (AA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>HTLLTPRR</td>
</tr>
<tr>
<td>A2</td>
<td>ICRLPUGC</td>
</tr>
<tr>
<td>A3</td>
<td>KAFWGLQH</td>
</tr>
<tr>
<td>A4</td>
<td>LALMLPGC</td>
</tr>
<tr>
<td>A5</td>
<td>LGFWGLPH</td>
</tr>
<tr>
<td>A6</td>
<td>LVCLPGC</td>
</tr>
<tr>
<td>A7</td>
<td>RALWGLQH</td>
</tr>
<tr>
<td>A8</td>
<td>SPWPPTY</td>
</tr>
<tr>
<td>A9</td>
<td>AKWGDITLCRWWR</td>
</tr>
<tr>
<td>A10</td>
<td>CIPMAVSHWPRI</td>
</tr>
<tr>
<td>A11</td>
<td>CIVWSDGKLRW</td>
</tr>
<tr>
<td>A12</td>
<td>CTYQYKCOELTA</td>
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<tr>
<td>A13</td>
<td>EWRLAEFLAMPP</td>
</tr>
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<td>A14</td>
<td>IAAHDTGPPWLS</td>
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<tr>
<td>A15</td>
<td>PNEVNRALHRLH</td>
</tr>
<tr>
<td>A16</td>
<td>SPLGYQFAVRNG</td>
</tr>
<tr>
<td>A17</td>
<td>YCAYSPHRKTF</td>
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**A8 and A17 are specific for inducible HSP70 and bind to distinct HSP70 domains**

The recently described small molecule inhibitor of HSP70, PES, binds to the peptide-binding domain of HSP70 (19), contrasting with the fact that HSP90 inhibitors that efficiently
block its chaperone activity (currently in clinical trials) bind to the ATP-binding domain of HSP90 (27). Immunoprecipitation experiments demonstrated that A17 binds to the HSP70 ATP-binding domain (HSP70\textsuperscript{D\textsubscript{PBD}}, and the aptamer A8 specifically binds to the HSP70 peptide-binding domain (HSP70\textsuperscript{D\textsubscript{ABD}}; Fig. 3A). To find the contribution of inducible HSP70 (as opposed to constitutive HSP70-like proteins) to A8- and A17-mediated cell killing, we evaluated chemosensitization by these aptamers on MEFs originating from wild-type mice or from mice that were deficient for inducible HSP70 (\textit{HSP70.1}, \textit{HSP70.3}). Both peptide aptamers A8 and A17 showed a strong chemosensitizing effect on wild-type MEFs responding to cisplatin (Fig. 3B). In sharp contrast, both aptamers were completely inactive on \textit{HSP70.1}\textsuperscript{\textminus\textminus} \textit{HSP70.3}\textsuperscript{\textminus\textminus} MEFs (Fig. 3B). Similarly, A8 and A17 lost their chemosensitizing properties in HeLa cells that were depleted from inducible HSP70 by small interfering RNAs (siRNAs; Fig. 3C). These results indicate that A8 and A17 both specifically exert their chemosensitizing effects through the blockade of the antiapoptotic activity of inducible HSP70.

The specific effect of A8 and A17 for the HSP70 chaperone was further studied by a novel method set up in our laboratory (Fig. 3D). Proteins were extracted from MEFs that lack \textit{HSF1}, the main transcription factor responsible for stress-induced HSP expression (28–30). Therefore, \textit{HSF1}\textsuperscript{\textminus\textminus} MEF cells express reduced levels of all inducible HSPs including HSP70. These \textit{HSF1}\textsuperscript{\textminus\textminus} MEF proteins were heated (55°C, 1 hour), and protein aggregation was determined in the presence or absence of recombinant HSPs, alone or in combination with the HSP70 peptides aptamers. By virtue of their chaperone activity, recombinant HSP70, HSC70, or HSP90 significantly reduced the amount of aggregated proteins (Fig. 3E and F). The A17 aptamer (and less so, the A8 aptamer) inhibited HSP70 chaperone activity, but no such inhibitory activity was observed for the control aptamer A0. Neither A17 nor A8 blocked the chaperone activity of recombinant HSC70 or HSP90 (Fig. 3E), indicating that they are indeed specific for HSP70.

We next tested, in this \textit{in vitro} assay, whether the synthetic heptapeptide (P8) and tridecapeptide (P17) corresponding to
the variable regions of A8 and A17, respectively, also inhibited the HSP70 chaperone activity. P17 was able to block HSP70 chaperone activity, yet failed to inhibit HSP90 (Fig. 3F). P8 exhibited a rather moderate inhibitory activity on the HSP70 chaperone function (Fig. 3F).

**HSP70-binding peptide aptamers induce tumor regression in vivo**

Next, we found whether A8 or A17 inhibited tumor growth in vivo. B16F10 melanoma cells were stably transfected with the aptamer expression vectors (A8, A17, and as a control, A0) and were injected subcutaneously into syngeneic C57/BL6 mice (9 mice/group). Stable expression of the peptide aptamers did not significantly alter the basal level of HSP70 or HSC70 (Fig. 4 and Supplementary Fig. 2). Cells expressing the A0 aptamer, such as wild-type B16F10 cells, formed tumors that rapidly progressed. In contrast, tumors expressing the aptamers A8 or A17 gave rise to smaller tumors that did not progress (Fig. 4A). Mice bearing B16F10-A0, B16F10-A8, or B16F10-A17 tumors were treated with cisplatin (10 mg/kg i.p.), given as a single dose on day 6 after the tumor cells injection. Cisplatin treatment slightly reduced the growth of control tumors (B16F10-A0), yet failed to eradicate these tumors. In contrast, most B16F10-A17 and, less so, B16F10-A8 tumors exhibited a complete response to cisplatin, and most mice remained tumor-free (8 mice of 9 for A17 and 6 of 9 for A8; Fig. 4A). Very similar results were obtained in the rat PROb colon cancer model (Supplementary Fig 3).

Interestingly, when B16F10-A8, B16F10-A17, and control B16F10-A0 melanoma cells were injected into immunodeficient athymic nude (nu/nu) mice, all tumors progressed with similar kinetics (Fig. 4B). Similarly, PROb-A0, PROb-A8, and PROb-A18 colon cancer cells proliferated indistinguishably in athymic nude rats (not shown). To further analyze the immune response induced by peptide aptamers, we conducted immunohistochemical analyses in sections from tumors grown in immunocompetent C57/BL6 mice. When compared with control B16F10-A0 tumors, B16F10-A8 and B16F10-A17 tumors exhibited a stronger infiltration by CD8+ T cells and macrophages (Supplementary Fig. 4 and Supplementary Table 1), suggesting that HSP70 inhibition in tumor cells can trigger an antitumor immune response (9, 21). This is
in accordance with our recent work, showing that HSP70 is abundantly expressed in the surface of exosomes secreted by cancer cells and is essential for the activation of the immuno-suppressive functions of myeloid cells (31).

**P17 sensitizes to apoptotic cell death in vitro**

HSP70 peptide aptamers synthesis/purification in sufficient quantities for their study in vivo in a more therapeutic, established tumor setting was difficult because of their poor solubility. Therefore, we decided to test whether we could use the 8- or 13-amino acid peptides from the variable region of the aptamers (Table 1). As mentioned above (Fig. 3F), the synthetic peptides P8 and P17, corresponding to the variable regions of A8 and A17, were able to inhibit the HSP70 chaperone activity in a cell-free assay. Further, the addition in the culture medium of P17 peptide (100 ng/mL), but not P8, was also able to sensitize B16F10 (Fig. 5A) and HeLa cells (not shown) to cisplatin-induced apoptosis. We concluded that although P8 lost the apoptosis-sensitizing properties of A8, P17 kept the HSP70 inhibitory and chemosensitizing properties of A17 (Figs. 3F and 5A). This differential effect might relate to the stability of peptides in the experimental medium. Although the peptides P0, P8, and P17 could be perfectly detected just after their addition in the culture medium (they gave the expected monoisotopic m/z values), only the P17 peptide was still detected by mass spectrometry analysis as late as 1 hour after its addition (Supplementary Fig. 5A). The increased stability of P17 might relate to its ability to associate with plasma lipoproteins (32, 33). Plasma lipoproteins were incubated for 1 hour at 37°C with HSP70 peptide aptamers, to protein extracts from HSF1+/− MEF cells. The mixture was heated at 55°C for 1 hour. The ratio between the amount of soluble proteins before and after heating allowed us to quantify protein aggregation. E, the inhibitory effect of A8 and A17 on HSP70 antiaggregation activity was quantified as described in D. F, the inhibitory effect of purified peptides P8 and P17 on HSP70 chaperone activity was measured as in D. Each bar is the mean value of 4 different experiments. *, P < 0.05.
were only detected in the lipoprotein-free/unbound fraction, P17 associated with plasma VLDL, LDL, and HDL (Supplementary Fig. 5C).

The chemical inhibitor of HSP70, PES, has been shown to induce autophagic cell death (19). We have, therefore, compared the cell death type induced by PES and P17 when combined with cisplatin treatment. We have found that although a clear vacuolization characteristic of autophagic cell death with the absence of caspase-3 activation could be observed in B16F10 cells sensitized to cisplatin by PES, no morphologic signs of autophagic cell death could be observed in the cells sensitized by P17 (Fig. 5B, right and left panels). In contrast, P17 induced the appearance of obvious signs of apoptosis (apoptotic bodies, chromatin condensation, and caspase-3 activity; Fig. 5B). We next studied whether P17 kept the ability of A17 to bind to the HSP70 ATP-binding domain. To do that, we linked the tridecapeptide to a biotin and carried out coimmunoprecipitation experiments in vitro with purified P17 and HA-tagged HSP70 or HSP70ΔPBD proteins. We found that P17-biotin was able to associate with both HSP70 and HSP70ΔPBD and, interestingly, when ATP (100 μmol/L) was added in the immunoprecipitation buffer, P17 maintained its binding ability (Fig. 5C). This may suggest that either ATP does not physically interfere with the P17 association with the HSP70 ATP-binding domain or, alternatively, that P17 binds with higher affinity than ATP.

Antitumor and immunogenic effect of P17 in tumor-bearing mice

We next tested the peptides P8 and P17 in intratumor injections in animals already bearing a tumor of approximately 90 mm³. Mice carrying B16F10 subcutaneous melanoma were injected intratumor with the P8 and P17 peptides (50 μg/kg, diluted in PBS). Peptide injections were repeated...
every day until the end of the experiment. Cisplatin (10 mg/kg; Fig. 6A) or 5-FU (50 mg/kg; Fig. 6B) was added i.p. as a single dose on the day after the first intratumor injection of the peptides. As shown in Figure 6A and B, local administration of P17, but not P8, induced a very significant regression of the tumors that was almost complete when the animals were also treated with cisplatin or 5-FU.

In a more therapeutic setting, we next administered the peptides systemically. Mice carrying B16F10 subcutaneous melanoma (tumor size of approximately 20–40 mm³) were injected i.p. (Fig. 7A) or i.v. (Supplementary Fig. 6) with P8 or P17 peptides (3 mg/kg). Peptide injections were repeated every other day. Half the animals were also treated with cisplatin (10 mg/kg), given i.p. as a single dose. P17 (but not P8) was efficient in reducing the size of the tumors, particularly when the tumors were growing in immunocompetent animals (Fig. 7A and Supplementary Fig 6) and less so when they were growing in athymic nu/nu mice (Fig. 7B), underscoring the importance of the immune system for the therapeutic efficacy of P17.

Immunohistochemical analyses of tumor sections 12 days after the first i.p injection of the peptides into tumor-bearing
mice confirmed that tumors from animals treated with P17 exhibited a stronger infiltration by T cells, macrophages, and dendritic and NK cells than tumors treated with P0 or P8 (Fig. 7C and Table 2). Since, in vitro, P17 sensitized to apoptotic cell death (Fig. 5A and B), we studied its ability, after its i.p. administration, to induce apoptosis in the tumor sections. P17 treatment significantly induced apoptosis in the tumors, whereas P8 hardly had any effect (Fig. 7D). Therefore, the amount of apoptosis in the tumors correlated well with the increase in immune cells infiltrating the tumor (Fig. 7C) and the magnitude of the tumor regression (Fig. 7A). Interestingly, although i.p. injection of P17 induced the tumor cells apoptosis, it has no significant toxicity in the mice intestinal progenitor cells, one of the most sensitive cells to different stresses (34; Supplementary Fig. 7).

In conclusion, P17 is an efficient antitumor agent. The fact that P17 but not P8 is active in vitro and in vivo may be related to its ability to bind plasma lipoproteins (Supplementary Fig 5 A–C). This association may protect the peptide from proteolysis. Indeed, and as compared with free peptides, plasma lipoproteins are known to reside much longer in the intravascular compartment with mean residence times reaching several days (35), and as a consequence, extending the half-life of lipoprotein-bound compounds in vivo. In addition, lipoprotein receptors might facilitate the cellular uptake of lipoprotein-bound peptides.

Concluding remarks

HSP-targeting drugs have recently emerged as potential anticancer agents, driven by the consideration that HSP may have oncogene-like functions and likewise may mediate a “nononcogene addiction” of stressed tumor cells that must adapt to a hostile microenvironment (36). Cancer cells must extensively rewire their metabolic and signal transduction pathways, thereby becoming dependent on proteins that are dispensable for the survival of normal cells. Unfortunately, the sole drugs that are thus far clinically available are inhibitors of HSP90, with most of them being geldanamycin derivatives such as 17AAG. We, and others, have validated HSP70 as a promising target for cancer therapy, using antisense molecules, a HSP70-binding construction derived from ALF (ADD70), and in this work, peptide aptamers that are specific for inducible HSP70. The cytotoxic effect of HSP70 sequestration is particularly strong in transformed cells yet is undetectable in normal, nontransformed cell lines or primary cells (8, 9). The specificity is explained by the constitutive expression of inducible HSP70 in most cancers, which is needed for the survival of tumor cells (37). This is confirmed in the present work since we have shown that inactivation of HSP70 by our peptide P17 induces apoptosis in tumor cells but does not affect the progenitors’ intestinal cells’ survival (Supplementary Fig. 7).

Unlike the recently reported PES (19), the aptamer that we isolated as the one to be most efficient in the inhibition of HSP70 chaperone activity, A17, binds to the HSP70 ATP-binding domain. HSP70 peptide aptamers did not have any toxicity on cultured cells in vitro yet strongly increased cellular sensitivity to toxic stimuli such as cisplatin. However, in vivo, the expression of the HSP70 peptide aptamers, or the injection of the derived peptide P17, was sufficient to induce tumor cell death. One possible explanation for this discrepancy might be that tumor cells growing in vivo are exposed to a more stressful microenvironment (i.e., lack of nutrients and/or oxygen) than cells cultured in vitro, explaining their increased dependence on (or “addiction” to) inducible HSP70.

The anticancer response induced by HSP70-targeting peptides, relied heavily on the contribution of the cellular immune system, as shown by the massive infiltration of macrophages, T lymphocytes, and dendritic and NK cells into the tumors.
treated by HSP70 peptide aptamers. This is an interesting observation that relaunches the debate on the immunogenic role of inducible HSP70 (38). Cytosolic HSP70 purified from distinct tumors can elicit tumor-specific immunity by functioning as a vehicle for antigenic peptides (39). The immunogenic and antiapoptotic functions of HSP70 may have opposite effects since tumor cell death plays a central role in inducing a specific immune response (40). However, we have recently shown that HSP70 expressed in the surface of exosomes produced by cancer cells is responsible for the activation of the immunosuppressive functions of myeloid cells (31). Therefore, inactivation of this external HSP70 can allow the induction of an immune antitumor response, such as the one we find in this work.

The pharmacologically most relevant finding of this work is the discovery that the 13-amino acid peptide (P17) from the variable region of A17 reproduces the HSP70-blocking chaperone activity and antitumor properties of A17 in vitro and in vivo. Because of their low molecular weight and high water solubility, small peptides are likely to be cleared within a few minutes from the bloodstream through renal filtration. Binding to lipoproteins can be a way of extending their half-life and to increase the cellular uptake (35). We have found that P17, but not P8, binds to plasma lipoproteins, correlating with its...
stability and antitumor efficacy. Therefore, P17, which can be easily synthesized and administered systemically, is a promising compound that may deserve further preclinical as well as clinical evaluation in phase I trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References


Table 2. Immunohistochemical analyses of tumor-infiltrating inflammatory cells in mice treated with P17

<table>
<thead>
<tr>
<th></th>
<th>B16F10-P0</th>
<th>B16F10-P0 + CDDP</th>
<th>B16F10-P8</th>
<th>B16F10-P8 + CDDP</th>
<th>B16F10-P17</th>
<th>B16F10-P17 + CDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c</td>
<td>0</td>
<td>0</td>
<td>4 ± 2</td>
<td>6 ± 3.5</td>
<td>17 ± 4</td>
<td>27 ± 4.8</td>
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<tr>
<td>F4/80</td>
<td>5 ± 3.5</td>
<td>3 ± 1.5</td>
<td>17 ± 3.9</td>
<td>19 ± 5.4</td>
<td>29 ± 4.8</td>
<td>47 ± 3.1</td>
</tr>
<tr>
<td>CD11b</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>37 ± 3.3</td>
<td>44 ± 5.4</td>
<td>44 ± 5.4</td>
</tr>
<tr>
<td>CD3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>23 ± 6</td>
<td>28 ± 3.9</td>
<td>28 ± 3.9</td>
</tr>
<tr>
<td>NKp46</td>
<td>0</td>
<td>0</td>
<td>3 ± 2</td>
<td>6 ± 1.5</td>
<td>15 ± 3</td>
<td>27 ± 5</td>
</tr>
</tbody>
</table>

NOTE: Quantitative evaluation of antigen expression in tumor sections 12 days after the first i.p. injection of P0, P8, or P17 into syngeneic mice. T cells, macrophages, monocytes, and dendritic cells were labeled using CD3, F4/80, CD11b, CD11c, and NKp46 antibodies, respectively. Labeled cells were counted from 300 cells chosen randomly in different microscopic fields (6 mice per group).

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Correction: Peptides and Aptamers Targeting HSP70: A Novel Approach for Anticancer Chemotherapy

In this article (Cancer Res 2011;71:484–95), which was published in the January 15, 2011, issue of Cancer Research (1), the author listing was inaccurate. The corrected author listing is below. The authors regret this error.


Reference
