A Simple Approach to Cancer Therapy Afforded by Multivalent Pseudopeptides That Target Cell-Surface Nucleoproteins

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Abstract

Recent studies have implicated the involvement of cell surface forms of nucleolin in tumor growth. In this study, we investigated whether a synthetic ligand of cell-surface nucleolin known as N6L could exert antitumor activity. We found that N6L inhibits the anchorage-dependent and independent growth of tumor cell lines and that it also hampers angiogenesis. Additionally, we found that N6L is a proapoptotic molecule that increases Annexin V staining and caspase-3/7 activity in vitro and DNA fragmentation in vivo. Through affinity isolation experiments and mass-spectrometry analysis, we also identified nucleophosmin as a new N6L target. Notably, in mouse xenograft models, N6L administration inhibited human tumor growth. Biodistribution studies carried out in tumor-bearing mice indicated that following administration N6L rapidly localizes to tumor tissue, consistent with its observed antitumor effects. Our findings define N6L as a novel anticancer drug candidate warranting further investigation. Cancer Res; 71(9); 3296–305. ©2011 AACR.

Introduction

Surface nucleolin expression is enhanced in various tumor cell lines and in activated endothelial cells (1–4). Furthermore, several molecules related to cell proliferation or cell differentiation have been reported to be ligands for cell-surface nucleolin. Among these molecules are hepatocyte growth factor, pleotrophin, and midkine, all of which play important roles in tumor development (5–8). In addition, molecules such as urokinase, which are involved in mechanisms regulating pericellular proteolysis and cell-surface adhesion mitogenesis, bind and are cointernalized with cell-surface nucleolin (9, 10). Other cell-surface nucleolin binding proteins, such as laminin-1, factor J, and L- and P-selectins, are involved in cell differentiation, cell adhesion regulation, leukocyte trafficking, inflammation, and angiogenesis (11–14). Cell-surface nucleolin has been validated as a novel target for anticancer therapy by using several molecules such as endostatin, the AS1411 aptamer, acharan sulfate, the F3 tumor-homing peptide coupled to radioactive isotopes (15–17), and the HB-19 pseudopeptide (18). Furthermore, targeting nucleolin with a specific antibody results in the activation of endothelial cell apoptosis by decreasing antiapoptotic Bcl-2 mRNA in the tumor vasculature (19).

In addition to its functions at the cell surface, nucleolin plays a critical role in the apoptotic process in which it regulates fundamental proteins such as Bcl-2, p53, and retinoblastoma. In chronic B-cell leukemia cells, overexpression of nucleolin has been directly linked to Bcl-2 mRNA stabilization, leading to apoptosis blockade and resistance (20, 21). In MCF-7 breast carcinoma cells and HeLa cells, a decrease in nucleolin level leads to an increase in p53 and apoptosis (22, 23). Nucleolin associates with tumor suppressor retinoblastoma protein, leading to cancer cell transformation (24).

In this study, we have investigated the antitumor activities, the mechanism of action, and the biodistribution in mice bearing human xenograft tumors of a synthetic ligand of cell-surface nucleolin known as N6L. In addition, using affinity isolation experiments and mass-spectrometry analysis, we identified other N6L molecular partners.
Materials and Methods

Peptide constructs
Synthesis of N4L, N5L, N6L, and N8L is described in detail in Supplementary Methods; briefly, the lysine-rich template is a 310 helical matrix composed respectively of 4, 5, 6, and 8 repeats of Lys-[CH2N]Pro-Arg, the pseudotripeptides Lys-[CH2N]Pro-Arg being grafted onto the ε NH2 of the matrix Lys residues. Fluorescent conjugates were prepared starting from the N6L-Cys derivative. The various conjugates were then purified by high-performance liquid chromatography and lyophilized.

Cell growth and toxicity assays
Cell growth was carried out as previously described (18) and cell viability measured and quantified by Cell Titer Aqueous One (MTS assay) according to the manufacturer’s instructions.

Chicken embryo chorioallantoic membrane assay
Chorioallantoic membrane (CAM) assay was carried out as previously described (18).

Tumor cell xenograft in nude mice
Experiments with human solid tumor xenografts in nude mice were carried out as previously described (18). For lymphoma experiments, A20 (5 × 106) or T29 lymphoma (5 × 106) was injected into BALB/c and C57BL/6 mouse tail vein after irradiation at 3 Gy/mice. Five days later, mice were randomly separated into 2 groups and treated with NaCl 0.9% (control group) or N6L 8 mg/kg 5 times per week by i.p. injection. All in vivo experiments were carried out with the approval of the appropriate ethical committee and under conditions established by the European Union.

In vivo tumor targeting of N6L
N6L-alexa fluor 633 (100 μg/mouse) was injected through the tail vein into nude mice bearing MDA-MB 231 xenograft tumors. Images were taken using the NightOwl Bioimager (Berthold Technologies). Signal intensity was evaluated using WinLight32 software.

Tissue preparation and apoptosis assay
Immediately after surgical resection, MDA-MB 231 tumors were frozen in liquid nitrogen and stored at −80°C. Fragmented DNA was stained using the ApopTag Isol Dual Fluorescent kit according to the manufacturer’s instructions (Millipore). Positive terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) cells were automatically counted by a program detecting the maximum of Sobel’s gradients, as previously described (25).

In vitro apoptosis assays
Cell death occurring in the presence of the N6L peptide was measured on a time scale of 30 minutes to 24 hours. Briefly, treated cells were harvested, rinsed, and resuspended in Annexin V-binding buffer containing allphophocyanin (APC)-labeled Annexin V (BD Bioscience) and propidium iodide (PI). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences). The decrease in the mitochondrial transmembrane potential was detected by the reduction of DiOC6 (3) dye uptake, and caspase-3/7 activity was measured using the Caspase-Glo 3/7 detection assay kit according to the manufacturer’s instruction (Promega).

Immunofluorescence staining and ApoTome microscopy
For immunofluorescence assays, after adding N6L-alexa fluor 488 0.5 μmol/L at 37°C for 30 minutes, cells were washed and fixed with cold acetone at −20°C for 10 minutes or with paraformaldehyde (PFA) 4% at room temperature for 10 minutes. Nucleolin and nucleophosmin were revealed using antinucleolin (MS-3, Santa Cruz Biotechnologies) and anti-nucleophosmin (Abcam) antibodies. DAPI 1 μg/mL was used for nuclear staining. All slides were observed with an AxioObserver Z.1 microscope equipped with an ApoTome system of structured illumination (Zeiss).

Short interfering RNA transfection
All binding experiments were conducted using the BIA CORE 3000 system and are described in detail in Supplementary Methods.

Statistical analysis
Statistical significance was determined by the ANOVA unpaired t test, using GraphPad Prism 4.0 software. Values of P < 0.05 were considered significant. Comparisons of survival curves were made using the Kaplan–Meier product-limit method and analysis by the log rank test. Statistical significance was defined as P < 0.05.

Results
Effect of N6L on tumor and endothelial cell survival and proliferation
We generated multivalent pseudopeptide analogues for use in exploring the most efficient size and shape of the template and the optimal number of grafted pseudotripeptides Lys-[CH2N]Pro-Arg. We then investigated the effects of these compounds on cell growth, using T29 cells. Comparative studies indicated that the optimum inhibitory effect was achieved with N6L (Fig. 1A). No effect of N6L was observed on the viability of BALB/c mouse lymph node cells used as a control (data not shown). N6L is built on a lysine-rich helicoid template formed by a repeat of 6 tripeptide units, (Lys-[CH2N]Pro-Arg being bound to the Lys ε NH2 groups of the template (Fig. 1B).

We then investigated the effect of N6L in a soft-agar colony-forming assay. In all cases, colony formation was reduced by

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50% or more, except for MDA-MB 231, in which 40% inhibition was observed (Fig. 1C and D).

Whatever the type of tumor cell, cell growth was significantly inhibited by N6L, with an inhibition of cell growth (GI50) value ranging from 5 to more than 40 μmol/L (Table 1). In addition, N6L significantly inhibited growth of human umbilical vein endothelial cells (HUVEC), with a 60% reduction at 10 μmol/L N6L (Fig. 2A). No effect was observed in cells treated with highly basic compounds such as polyarginine. In vivo angiogenic assay, addition of N6L impaired the branching of newly formed blood vessels in comparison with the PBS control (Fig. 2B). Quantitative analysis indicated that the maximal inhibition of angiogenesis was reached with the application of 400 pmol of N6L.

**Antitumor effect of the multivalent pseudopeptide N6L**

To determine the in vivo efficacy of N6L as an antitumor molecule, we carried out several experiments with mice bearing human xenograft tumors and murine lymphoma. Compared with the control, N6L induced significant inhibition of tumor growth in mice grafted with MDA-MB 231 or PC3 cells. Indeed, N6L used at concentrations as low as 1 mg/kg inhibited tumor growth up to 90% (P < 0.001) and 40% for MDA-MB 231 and PC3 cells, respectively (Fig. 2C and D). It is worth mentioning that N6L efficacy on MDA-MB 231 is optimal between low and high doses, which may reveal a biphasic dose response as already observed for other regulators of cell growth (26).

Similar results were obtained by analyzing the weights of tumors harvested from tumor-bearing mice (Supplementary Fig. S1). Consistent with in vitro observations (Table 1), the survival of N6L-treated mice bearing A20 and T29 lymphomas was significantly prolonged compared with controls (Fig. 2E and F). We found no apparent toxicity in several organs of N6L-treated mice and did not observe significant changes in their body weight compared with control mice (data not shown). These results indicate that...
N6L effectively reduced tumor growth in mice without evidence of toxicity.

**Identification of N6L cell targets**

To identify molecular targets of N6L, we undertook a series of affinity isolation experiments based on a previously described method (27) using Raji and MDA-MB 231 cells and biotin-labeled N6L. Affinity isolated proteins were subjected to SDS-PAGE and analyzed by mass spectrometry (Supplementary Table S1). This procedure enabled us to identify several proteins involved in ribonucleoprotein complexes, including nucleolin and nucleophosmin (Fig. 3A). This result was validated by Western blot experiments (Fig. 3B). Analysis of the equilibrium binding parameters using surface plasmon resonance experiments indicated that both nucleolin and nucleophosmin are usually found to be associated with RNA in the ribosome biogenesis pathway, we investigated whether the interaction between nucleolin or nucleophosmin and N6L is direct or dependent on a bridging molecule like RNA. Treatment of the affinity isolated fraction with a large excess of RNase revealed that nucleolin—but not nucleophosmin—interactions with N6L were RNA-mediated (Fig. 3B). This indicated that, whereas N6L is able to interact with both nucleolin and nucleophosmin, RNA could modulate nucleolin structure to promote N6L binding to nucleolin. These results are reinforced by data from cell-surface biotinylation experiments which provided evidence on the presence of nucleophosmin and nucleolin at the tumor cell surface (Fig. 3D), as well as on the colocalization of N6L with nucleophosmin in nonpermeabilized MDA-MB 231 cells (Fig. 3E).

**N6L exhibits a proapoptotic activity in vitro**

We used video microscopy to carry out direct analysis of the metabolism of the N6L pseudopeptide. MDA-MB 231 cells were incubated in the presence of N6L-alexa fluor 488 and analyzed for 14 hours by time-lapse video microscopy. Treated cells rapidly rounded and revealed morphologic features of characteristic apoptosis, such as cell shrinkage, cell membrane blebbing, and formation of apoptotic bodies (Fig. 5A and Supplementary Movie).

To validate these observations, apoptosis of N6L-treated cells was quantified by flow cytometry using Annexin V/PI and DIOC6 staining. N6L markedly increased Raji cell apoptosis in a concentration-dependent (Fig. 5B and C) and time-dependent manner (Fig. 5D). Similar data were obtained using MDA-MB 231 and MDA-MB 435 cells (Supplementary Table S2). To further confirm that N6L-induced cell death was due to apoptosis, we analyzed caspase-3/7 activation, which is considered...
to be a common hallmark of apoptosis. Raji cells were treated with concentrations of N6L ranging from 0 to 50 μmol/L for 48 hours and caspase-3/7 activity was determined in the cell lysate. N6L was found to induce caspase-3/7 activation in a dose-dependent manner. A 2-fold increase in caspase-3/7 activity compared with the basal level was observed in cells treated with 20 μmol/L N6L. (Fig. 5E).

In vivo tumor targeting of N6L
Mice bearing MDA-MB 231 tumors were i.v. injected with 100 μg of N6L-alexa fluor 633 and subjected to fluorescence tracking in a bioimager. Images were collected every 2 minutes over the first 20 minutes, then every 24 hours. N6L-alexa fluor 633 revealed wide tissue distribution, including tumor tissue, immediately after injection, thus demonstrating the rapid addressing of the peptide due to efficient perfusion (Fig. 6A). A persistent signal in the tumor and in various other organs, including lung, kidney, liver, and brain, was observed. Postmortem collection of these tissues confirmed the retention of N6L-alexa fluor 633 24 hours postinjection. The signal intensity progressively decreased in all organs while increasing in the tumor mass until 48 hours postinjection, revealing the natural enrichment of the N6L pseudopeptide in tumors (Fig. 6B). Interestingly, persistent enrichment of N6L-alexa fluor 633 was also observed in the tumor mass in case of repeated injections (every day for 3 days; Fig. 6C). In addition, and as a control, we also monitored the distribution of N6L-alexa fluor 633 in mice without implanted tumor cells. In this case, we observed widespread distribution with most of the pseudopeptide being in lung, kidney, and liver, whereas no detectable signal was found in spleen and brain (Supplementary Fig. S2A and B). An additional control was carried out with the inactive N6L-alexa fluor 633 template in mice bearing tumors. A widespread distribution was also observed with no detectable fluorescence enrichment in the tumor mass (Supplementary Fig. S5).

N6L mediates caspase-dependent apoptosis in vivo
Sections of tumor xenografts from MDA-MB 231 cells treated or not with N6L were subjected to TUNEL staining (Fig. 5F). This staining causes a red fluorescence to appear that corresponds to a 5’PO4 DNA fragment cleaved by type I DNAse activated by caspase-3 and involved in self-execution apoptosis, as well as a green fluorescence that corresponds to an independently activated 5’-OH DNA fragment cleaved by type II DNAse caspase. Low red and green fluorescence was
observed in control tumors, indicating that the number of apoptotic cells was very low (relative score of 1,911/C6 487, n = 5). In N6L-treated tumors, strong red signals (relative score of 35,580/C6 8,553, n = 5)—but no green ones—were observed.

Comparison of the relative scores indicated that the inhibition of tumor growth in athymic mice by N6L occurred through the induction of caspase-dependent cell apoptosis (P < 0.001). Similar results were obtained in experiments carried out using xenograft PC-3 cells (Supplementary Fig. S3).

Discussion

The results presented in this study demonstrate the antitumor activity of a new multivalent pseudopeptide called N6L. Using an optical imaging system, this effect was validated in vivo, showing that N6L specifically targeted the tumor. These experiments were carried out in mice bearing MDA-MB 231 (Fig. 6A) or U373MG tumors (Supplementary Fig. S4). One of the major result of this study is the demonstration that N6L promotes apoptosis, as shown in both in vitro and in vivo experiments. Induction of apoptosis by N6L was directly observed by video microscopy in N6L-treated cells that exhibited abnormal cell morphology characteristic of apoptosis (28). Nucleolin has been identified as a Bcl-2 mRNA-stabilizing protein in the HL-60 human leukemia cell line (20, 29). More recently, it has been shown that the resistance of B-cell lymphocytic leukemias to apoptosis as a result of Bcl-2 gene overexpression is related to stabilization of Bcl-2 mRNA by
In addition, siRNA knockdown of nucleolin resulted in decreased Bcl-2 mRNA stability and decreased levels of Bcl-2 protein in MCF-7 cells (30). Interestingly, several nucleolin ligands were shown to be involved in the induction of apoptosis. For example, the nucleolin-targeting aptamer AS1411 has been shown to induce apoptosis by interfering with the binding of nucleolin to Bcl-2 mRNA (30). Similarly, antinucleolin antibody treatment destabilizes Bcl-2 mRNA, leading to endothelial cell apoptosis (19). Endostatin, a 20-kDa polypeptide that is a potent inhibitor of angiogenesis and tumor growth (31), binds specifically cell-surface nucleolin (17). Study of the mechanism of action of endostatin on tumor growth has indicated that it induces apoptosis through activation of caspase-3 and a decrease in Bcl-2 mRNA (32, 33). Taken together, these observations indicate that cell-surface nucleolin is highly involved in the apoptosis process, which is also supported by the present results showing that N6L is a proapoptotic molecule able to induce tumor cell death.

Although cell-surface nucleolin is a good candidate for N6L-mediated apoptosis induction, we cannot exclude that other N6L-binding proteins and/or nucleolin-binding proteins could be involved and might play a role in this process. To test this hypothesis, a series of affinity isolation experiments were carried out. As expected, nucleolin was identified, but also several other proteins, including ribosomal proteins and signal recognition particle proteins involved in the packing and transport of RNA, as well as nucleophosmin. Because all the proteins identified in this experiment belong to a protein family that shares the common property of being able to bind nucleolin, we can assume that most of these affinity-isolated proteins are associated in a macromolecular complex that includes cell-surface nucleolin (34). Among these proteins, nucleophosmin is an important target for cancer suppression and is involved in apoptosis regulation. Studies using a peptide derived from Rev protein, which binds nucleophosmin, suppressed tumor growth through apoptosis by upregulating the transcriptional activity of p53. Recently, NSC348884, a pharmacophore that targets nucleophosmin, has been shown to inhibit the proliferation of various cancer cell lines through the induction of apoptosis (35). Likewise, it has been shown that
both nucleophosmin and nucleolin, which are overexpressed at the cell surface of tumor cells compared with normal resting cells (36), regulate K-Ras plasma membrane interactions and mitogen-activated protein kinase signal transduction. Analysis of the binding domains involved in these interactions revealed that nucleophosmin binds to the basic domain of K-Ras. This stretch of basic residues interacts with the N-terminal globular domain of nucleophosmin, which has been shown to be involved in nucleophosmin pentamer formation and chaperone activity. It is noteworthy that molecules that interact with the N-terminal domain of nucleophosmin, such as the Rev peptide (37) or the small molecule NSC 348884 (35), disrupt

Figure 5. N6L induces apoptosis in treated tumors cells. A, analysis of the biological effect of N6L by time-lapse video-microscopy analysis. Ten μmol/L of N6L alexa-fluor 488 was added to MDA-MB 231 cells. Nuclei were stained with Hoechst reagent. Pictures were taken with a time-lapse system every 10 minutes for 14 hours. Arrows indicate the evolution of one cell. Scale bars represent 10 μm. B, N6L (0–40 μmol/L for 16 hours) induces a decrease of mitochondrial Δm in Raji cells, measured by the reduction of DIOC6 dye uptake, early apoptosis measured by Annexin V–allophycocyanin-labeling, and morphologic changes relative to apoptosis shown in an SSC/FSC plot obtained by flow cytometry. C, percentage of early (Annexin V+/PI−) and late apoptotic cells (Annexin V+/PI+) measured in flow cytometry during N6L-induced apoptosis of Raji cells after 16 hours of treatment. D, time-course of apoptosis induction of Raji cells by N6L peptide at increasing concentrations. Results are expressed as percentage of specific apoptosis according to the following formula: percentage specific apoptosis = (percentage of apoptotic treated cells – percentage of apoptotic control cells) × 100/(100 – percentage of apoptotic control cells). E, caspase-3/7 activity in Raji cells treated with different concentrations of N6L or by camptothecin as a positive control for 24 hours; RLU, relative light units. F, N6L induces caspase-dependant apoptosis in vivo in MDA-MB 231 tumors. Red signal corresponds to type I DNAse activation-dependent on caspase-3 activation. Green signal corresponds to type II DNAse activation-independent of caspase activation. Scale bar represents 50 μm.
nucleophosmin oligomer formation and induce apoptosis. From this set of results, we can hypothesize that N6L, which is a pseudopeptide rich in Lys and Arg residues and displays high affinity for nucleophosmin, disrupts its oligomerization and/or the formation of complexes between nucleolin, nucleophosmin, and oncoproteins. Another piece of data presented herein concerns cellular trafficking of N6L to the tumor cell nucleolus. Following its binding to cell-surface nucleoproteins, N6L is internalized and rapidly concentrates in the nucleolus. This result was obtained using fluorescent video-microscopy analysis on live cells and was further confirmed by histologic studies using N6L tagged with fluorescent molecules. The mechanism by which N6L is translocated to the nucleolus remains unknown and is currently under investigation. However, it is tempting to speculate that N6L could be translocated to the nucleolus through its interaction with nucleoproteins. These results are supported by our experiments on N6L trafficking on nucleolin and nucleophosmin knockdown cells. Nevertheless, the relationship between localization of N6L in the nucleolus and its biological effect has not yet been established and will be explored in further studies.

Viewed collectively, the data presented in this article indicate that the multivalent pseudopeptide N6L induces apoptosis of tumor cells, which could therefore constitute a possible mechanism by which N6L demonstrates antitumor activity. Both nucleolin and nucleophosmin are multifunctional pleiotropic proteins involved in several important cell processes, suggesting that the antiproliferative effect of N6L could be mediated by several different mechanisms of action that remain to be identified.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank M. Duval and S. Besse for their valuable assistance, G. Courty for the video editing, and Dr P. Kastner (IGBMC, Strasbourg) for the gift of T29 cells.

Grant Support

This research was funded by the CNRS, the Région Alsace, La Ligue contre le Cancer, and the Ministère de l’Enseignement Supérieur et de la Recherche.

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Received September 23, 2010; revised February 24, 2011; accepted March 9, 2011; published OnlineFirst March 17, 2011.
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Cancer Res 2011;71:3296-3305. Published OnlineFirst March 17, 2011.

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