Cancer Letters 412 (2018) 272–282

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Article

N6L pseudopeptide interferes with nucleophosmin protein-protein interactions and sensitizes leukemic cells to chemotherapy

A. De Cola [a], M. Franceschini [a], A. Di Matteo [b], G. Colotti [b], R. Celani [a], E. Clemente [a], R. Ippoliti [c], A.M. Cimini [c, d, e], A.C. Dhez [c], B. Vallée [f], F. Raineri [f], I. Cascone [f], D. Destouches [f], V. De Laurenzi [a], J. Courty [f], L. Federici [a, *]

[a] Dipartimento di Scienze Mediche, Orali e Biotecnologiche, CESI-MeT, Centro Scienze dell'Invecchiamento e Medicina Traslazionale, Universita' "G. d'Annunzio" Chieti-Pescara, Chieti, Italy
[b] Istituto di Biologia e Patologia Molecolari del CNR, Rome, Italy
[c] Dipartimento di Medicina Clinica, Sanità Pubblica, Scienze della Vita e dell'Ambiente, Università dell'Aquila, L'Aquila, Italy
[d] Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, Temple University, Philadelphia, USA
[e] National Institute for Nuclear Physics (INFN), Gran Sasso National Laboratory (LNGS), Assergi, Italy
[f] Universite; Paris-Est Créteil, CNRS, ERL 9215, Laboratoire de Recherche sur la Croissance Cellulaire, la Reparation et la Regeneration Tissulaires (CRRET), Creteil, F-94000, France

ARTICLE INFO

Article history:
Received 1 June 2017
Received in revised form
24 October 2017
Accepted 24 October 2017

Keywords:
NPM1 mutations
Acute myeloid leukemia
Targeted therapy

ABSTRACT

NPM1 is a multifunctional nucleolar protein implicated in several processes such as ribosome maturation and export, DNA damage response and apoptotic response to stress stimuli. The *NPM1* gene is involved in human tumorigenesis and is found mutated in one third of acute myeloid leukemia patients, leading to the aberrant cytoplasmic localization of NPM1. Recent studies indicated that the N6L multivalent pseudopeptide, a synthetic ligand of cell–surface nucleolin, is also able to bind NPM1 with high affinity. N6L inhibits cell growth with different mechanisms and represents a good candidate as a novel anti-cancer drug for a number of malignancies of different histological origin. In this study we investigated whether N6L treatment could drive antitumor effect in acute myeloid leukemia cell lines. We found that N6L binds NPM1 at the N-terminal domain, co-localizes with cytoplasmic, mutated NPM1, and interferes with its protein-protein associations. N6L toxicity appears to be p53 dependent but interestingly, the leukemic cell line harbouring the mutated form of NPM1 is more resistant to treatment, suggesting that NPM1 cytoplasmic delocalization confers protection from p53 activation. Moreover, we show that N6L sensitizes AML cells to doxorubicin and cytarabine treatment. These studies suggest that N6L may be a promising option in combination therapies for acute myeloid leukemia treatment.

Introduction

Nucleophosmin (NPM1) is a nucleolar phosphoprotein expressed in all tissues [1]. It is considered one of the hub proteins of the nucleoli where it plays structural and functional roles through the interaction with several protein and nucleic acid partners [2,3]. NPM1 is involved in several crucial cellular activities including: centrosome duplication [4], rRNA biogenesis and maturation [5], DNA repair [6] and chaperone activity [7]. NPM1 alterations also appear to be involved in cancer development [8,9]. Indeed NPM1 overexpression has been reported in several solid tumors including prostate [10], liver [11], thyroid [12], colon [13], gastric [14], pancreas [15], glioma and glioblastoma [16,17] and often correlates with increased mitotic index and metastasization. The role of NPM1 is also relevant in haematological malignancies where it is often found translocated, deleted or mutated [18]. Notably, *NPM1* is the most frequently mutated gene in AML (acute myeloid leukemia) patients [19,20]. Mutations identified so far are always heterozygous and localized in the terminal exon of the gene [20]. Frameshift insertions cause: the unfolding of the protein C-terminal domain [21]; loss of affinity for nucleic acids [22,23]; loss of the nucleolar localization sequence and the appearance of a new

* Corresponding author. Dipartimento di Scienze Mediche, Orali e Biotecnologiche, CeSI-MeT Centro Scienze dell'Invecchiamento e Medicina Traslazionale, Università di Chieti-Pescara "G d'Annunzio", Via dei Vestini 31, 66100 Chieti, Italy.
E-mail addresses: lfederici@unich.it, luca.federici@unich.it (L. Federici).

https://doi.org/10.1016/j.canlet.2017.10.038
0304-3835/

nuclear export signal [19,24]. As a result, mutated NPM1 is found stably and aberrantly localized in the cytoplasm and it is therefore indicated as NPM1c+. Since the protein heterodimerizes through its N-terminal domain, also wild-type NPM1 is mostly cytoplasmic and only a small fraction of NPM1 is retained in nucleoli in the presence of the mutated form [19,20].

The mechanism by which NPM1c + exerts its tumorigenic activity is not yet completely understood but it has been suggested that the "wrong" cytoplasmic localization may interfere with tumor suppressive mechanisms at multiple levels. For instance NPM1c + interacts with and translocates p14ARF to the cytosol leading to its degradation and thus impairing the p14ARF-HDM2-p53 axis [25]. The same mechanism of delocalization and degradation also applies to the tumor suppressor Fbw7γ, the nucleolar E3-ubiquitin ligase of c-MYC, whose half-life is thus increased in leukemic cells [26]. Furthermore NPM1c + interacts with and inhibits the PTEN deubiquitinating enzyme HAUSP, resulting in PTEN polyubiquitination and degradation [27]. Taken together these studies suggest that moving a critical regulator to the wrong cellular compartment may confer a survival advantage to leukemic cells [28]. These data led us to suggest that new therapeutic strategies aimed at targeting NPM1c + protein-protein associations may be useful for AML treatment [29].

Recently, NPM1 has been identified as one of the targets of the anticancer molecule NucAnt 6L (N6L) [30]. N6L is a synthetic multimeric pseudopeptide, rich in lysine and arginine residues, which has successfully completed phase I/IIa and is currently in preparation for phase II clinical trials. N6L specifically binds a nucleolin-receptor complex overexpressed selectively at the cell surface of tumor cell lines derived from melanoma, glioblastoma, mammary and colorectal carcinoma [30] and, after internalization, mediates anti-tumor activities. N6L inhibits the growth of tumor cell lines, hampers angiogenesis, exerts pro-apoptotic activity *in vitro* and rapidly localizes to tumor tissues *in vivo* [31–36]. N6L has been developed as a nucleolin ligand, however its interaction with NPM1 could be responsible, at least in part, for its anti-tumor activities.

Here we investigated the interaction of N6L with NPM1 and the effect that N6L plays in an AML cell line harbouring NPM1c+, in comparison with an AML cell line where NPM1 is wild-type. We show that N6L interacts with NPM1 N-terminal domain, interferes with NPM1 protein-protein associations and co-localizes in the cytosol with NPM1c+. N6L exerts its toxicity in AML cell lines *via* p53 activation but this effect is strongly delayed in the AML cell line expressing NPM1c+, suggesting that NPM1 delocalization confers resistance to p53 activation. However and importantly, our data also point out that N6L sensitizes AML cells harbouring NPM1c + to both doxorubicin and cytarabine, which are commonly used in AML treatment. Thus, we suggest that the efficacy of N6L may be further explored in combination studies aimed at those AML patients that cannot sustain treatment with either of the two drugs.

Material and methods

SPR analysis

Surface Plasmon Resonance (SPR) experiments were carried out with a SensiQ Pioneer apparatus. Streptavidin-coated BioCap sensorchips were chemically activated by a 35 μl injection of 10 mM NaOH at 10 μl/min flow rate. Biotinylated N6L was immobilized *via* interaction with the streptavidin surface of the chips. The amount of immobilized N6L was detected by mass concentration-dependent changes in the refractive index on the sensor chip surface, and corresponded to about 50 resonance units in each experiment (RU).

Binding experiments were carried out at 298 K in degassed 20 mM HEPES at pH 7.0, 0.15 M NaCl, and 0.005% surfactant P-20 (HBS-P buffer). For both human full-length (FL; residues 16-294) NPM1 and its isolated N-terminal domain (NPM1-Nter; residues 16-123), injections were carried out as follows. The proteins were automatically diluted in HBS-P and injected by 7 serial doubling steps (step contact time = 25 s, nominal flow rate = 100 μl/min), at the following time points: 1) 0–25 s; 2) 26–50 s; 3) 51–75 s; 4) 76–100 s; 5) 101–125 s; 6) 126–150 s; 7) 151–162 s. FL NPM1 concentrations were: 1) 0.375 μM; 2) 0.75 μM; 3) 1.5 μM; 4) 3.0 μM; 5) 6.0 μM; 6) 12 μM; 7) 24 μM and 1) 0.0625 μM; 2) 0.125 μM; 3) 0.25 μM; 4) 0.5 μM; 5) 1.0 μM; 6) 2.0 μM; 7) 4.0 μM; NPM1-Nter concentrations were: 1) 2.34 μM; 2) 4.69 μM; 3) 9.38 μM; 4) 18.75 μM; 5) 37.5 μM; 6) 75 μM; 7) 150 μM and 1) 0.68 μM; 2) 1.55 μM; 3) 3.13 μM; 4) 6.25 μM; 5) 12.5 μM; 6) 25 μM; 7) 50 μM. The increase in RU relative to baseline indicates complex formation between the immobilized N-biotinylated peptide and NPM1 constructs. The plateau region represents the steady-state phase of the interaction. The decrease in RU after 162 s for FL and NPM1-Nter indicates protein dissociation from the immobilized peptide after buffer injection. A response change of 1000 RU typically corresponds to 1 ng/mm^2 change in analyte concentration on the sensor chip. As a negative control, sensor chips were treated as described above in the absence of immobilized N-biotinylated peptides. Values of the plateau signal at steady-state (R_{eq}) and full fittings with 1, 2 and 3 sites were calculated from kinetic evaluation of the sensorgrams using the Qdat 4.0 program.

Fluorescence analysis

Competition experiment was performed at 25 °C in sodium phosphate buffer 20 mM pH 7.2. FluoroMax-4 spectrofluorometer (Jobin Yvon, Edison, NJ, USA), equipped with a water bath apparatus, was used to collect data. NPM1 N-terminal domain and a dansylated peptide of sequence LPFCRRRMKRKLDH, representing the predicted nucleolar localization signal of the Fbw7γ protein (JPT, Germany) were mixed at equal amounts in quantities amenable to ensure complete complex formation (20 μM each). This mixture was titrated with N6L as reported in Fig. 1C. Excitation wavelength was 330 nm and emission spectra were collected in the range between 350 and 650 nm, to monitor dansyl fluorescence.

Cell culture

Acute myeloid leukemia cells (AML-OCI-2 and AML-OCI-3) were grown in MEM-alpha-Medium with high glucose (GibcoBRL). Cell lines were grown in suspension at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air. All the media were supplemented with 20% (v/v) fetal bovine serum (FBS) (Euroclone).

Infections

Lentiviruses were produced by transient cotransfection of a three-plasmid expression system in the packaging 293T cells, using the calcium phosphate transfection kit (Invitrogen, Life Technologies). The lentiviral vectors shp53 pLKO.1 puro and scramble shRNA were purchased from Addgene.

Cells were incubated for 7 h with the transfection reagents and viral supernatant was collected 48 h after transfection and filtered through 0.45 μm pore vacuum sterile filtration system (Millipore). Then, AML cells were plated in a six-well plate (Corning) with viral supernatant and 4 μg/mL of polybrene. Plates were centrifuged for 45′ at 1800 rpm and incubated at 37 °C for 75′ in a 5% CO_2 humified chamber. Cells were then washed twice and replated in fresh medium. Cells were selected by puromycin treatment and then infection efficiency was assessed by western blot analysis anti-p53.

Western blotting

Proteins were extracted with a lysis buffer (TRIS-HCl 50 mM pH 8, NaCl 150 mM, Triton X-100 1%, NaF 100 mM, EDTA 1 mM, $MgCl_2$ 1 mM, Glycerol 10%) containing a protease inhibitor cocktail (Sigma-Aldrich) and a phosphatase inhibitor cocktail (Roche). Equal amounts of total protein were subjected to SDS-PAGE and then electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS with 0,1% Tween 20 and incubated over-night using the following antibodies: anti β-Actin A5441 (Sigma), anti-NPM1, anti-Fibrillarin, anti-p14 ARF (Cell Signaling), anti-p21 and anti-p53 (Santa Cruz), anti-Nucleolin (Upstate, Millipore) anti-NPM1 Thr199 and Thr234/237 (Biolegend). After wash, membranes were hybridized with horseradish peroxidase-conjugated secondary antibodies (rabbit and mouse, Biorad, CA, USA). Detection was performed with Plus-ECL chemiluminescence kit (PerkinElmer, Inc.; MA, USA) or with SuperSignal West Dura extended duration substrate kit (Thermo Scientific, USA).

In vitro apoptosis assays

Apoptotic cell death in the presence of 20 μM N6L for 24 h and 48 h was evaluated by Annexin V staining. Briefly cells were resuspended in Annexin V binding buffer containing allophycocyanin (APC)-labeled Annexin V (BD Biosciences) and 7-Amino-Actinomycin D (7-AAD) as a viability probe. A FACSCantoII flow cytometer, running with FACSDiVa software (BD Biosciences), was used for sample acquisition and analysis.

Caspase-3/7 activity was measured using the Caspase-Glo 3/7 detection assay kit according to the manufacturer's instruction (Promega) with Synergy H1 Gen5 microplate reader and Imager (Biotek).