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Novel phage display-derived neuroblastoma-targeting peptides potentiate the effect of drug nanocarriers in preclinical settings ☆

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ABSTRACT

Molecular targeting of drug delivery nanocarriers is expected to improve their therapeutic index while decreasing their toxicity. Here we report the identification and characterization of novel peptide ligands specific for cells present in high-risk neuroblastoma (NB), a childhood tumor mostly refractory to current therapies. To isolate such targeting moieties, we performed combined *in vitro*/*ex-vivo* phage display screenings on NB cell lines and on tumors derived from orthotopic mouse models of human NB.

By designing proper subtractive protocols, we identified phage clones specific either for the primary tumor, its metastases, or for their respective stromal components. Globally, we isolated 121 phage-displayed NB-binding peptides: 26 bound the primary tumor, 15 the metastatic mass, 57 and 23 their respective microenvironments. Of these, five phage clones were further validated for their specific binding ex-vivo to biopsies from stage IV NB patients and to NB tumors derived from mice. All five clones also targeted tumor cells and vasculature *in vivo* when injected into NB-bearing mice. Coupling of the corresponding targeting peptides with doxorubicin-loaded liposomes led to a significant inhibition in tumor volume and enhanced survival in preclinical NB models, thereby paving the way to their clinical development.

1. Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in children, accounting for about 8% of childhood cancers. Approximately 40% of NB tumors are classified as high-risk; their management includes combinations of chemotherapy, autologous stem cell transplantation, surgery, and radiation therapy. Despite this aggressive treatment, children with high-risk NB have very poor 5-year overall survival rate, due to relapsed and/or treatment-resistant tumors [1,2]. A further increase in therapeutic dose intensity is not feasible, because it will lead to prohibitive short-term and long-term toxicities. New approaches with targeted therapies may improve efficacy and decrease toxicity [3].

Extensive investigation of NB biology has recently resulted in the identification of a wide range of potential druggable targets, most of which are commonly deregulated in different solid tumors, e.g., disialoganglioside GD_2 [3], anaplastic lymphoma kinase (ALK) [4], aurora kinase A (AURKA) [5], epidermal growth factor receptor (EGFR) [6], insulin growth factor 1 receptor (IGF1R) [7], mammalian target of rapamycin (mTOR) [7], and vascular endothelial growth factor receptor 2 (VEGFR2) [8]. Several compounds have proved to be highly active in preclinical models, and at least 15 genes are presently in the clinical pipeline as targets for NB therapy [9]. However, small

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molecule inhibitors and blocking antibodies suffer from a number of limitations, including toxic side effects, induction of resistance, and low response rates. Together, reports from the literature and from clinical trials provide evidence that, unfortunately, for more than 50% of patients with high-risk NB there are substantially no curative options available.

The use of drug delivery systems allows site specific delivery of higher payload of active agents associated with lower systemic toxicities compared to the use of conventional ('free') drugs; the possibility of imparting selectivity to the carrier to the cancer foci through the use of a targeting moiety (e.g., a peptide or an antibody) further enhances drug efficacy and safety [10–13]. The peptide motifs, NGR [14] and CPRECES [15], home to the vasculature of different tumor types and we have recently exploited them to vehiculate chemotherapeutic agents to tumor blood vessels in preclinical models of NB [16–18]. However, NB-targeting peptides with greater selectivity, which are expected to substantially improve current therapeutic regimens, have not been identified thus far.

We report here the identification of novel peptide ligands, selected by combined *in vitro*/*ex-vivo* phage display screenings in preclinical models of human NB. Five sequences with differential specificity for either epithelial or stromal components of the tumors were validated *in vitro* and *in vivo* for their targeting specificity. The capability of peptide-targeted drug delivery systems to counteract tumor progression was investigated in biologically relevant murine models of human NB. We demonstrated that this approach allows a substantial improvement in therapeutic efficacy compared to both free drug and untargeted systems.

2. Materials and methods

2.1. Cells lines and human samples

The neuroblastoma (NB) cell lines (GI-LI-N, HTLA-230 and IMR-32) were grown in complete Dulbecco's Modified Eagle Medium (DMEM) or RPMI-1640 medium, as previously described [16,19]. Human umbilical vein endothelial cells (HUVECs) were maintained in endothelial cell basal medium-2 (Cambrex Bio Science), as described [20]. Cells were tested for mycoplasma contamination and characterized by cell proliferation, morphology evaluation, and multiplex short tandem repeat profiling test. Human samples (07-B-822, 07-B-1173 and 07-B-1312A2) derived from stroma poor, stage IV, NB patients, were provided, after informed consent, by Bio-bank, Istituto Giannina Gaslini, Genoa, Italy.

2.2. Animal models

Animals were purchased from Harlan Laboratories (Harlan Italy, S. Pietro al Natisone, Italy) and were housed under pathogen-free conditions; experiments were reviewed and approved by the licensing and ethical committee of IRCCS Azienda Ospedaliera Universitaria San Martino — IST Istituto Nazionale per la Ricerca sul Cancro (Genoa, Italy), and by the Italian Ministry of Health. For the orthotipic model, 5-week-old athymic (nu/nu) female mice were injected with 1×10^6 GI-LI-N or HTLA-230 cells in the left adrenal gland, as described [16]. No mice died as a result of the surgery. Animals were monitored at least twice a week for evidence of tumor development and quantification of tumor size, and were sacrificed by cervical dislocation after being anesthetized with xilezine (Xilor 2%, Bio98 Srl, Milan, Italy), when they showed signs of poor health, e.g. abdominal dilation, dehydration, or paraplegia. For the pseudometastatic model, 4-week-old female athymic (nu/nu) mice were injected intravenously (*i.v.*) in the tail vein with 4×10^6 HTLA-230 cells, as previously described [19]. Body weight and general physical status were recorded daily, and mice were sacrificed by cervical dislocation after the administration of xilezine, when they showed signs of poor health.

2.3. Phage display biopanning on preclinical models of human neuroblastoma

Cultured GI-LI-N and HTLA-230 cells were detached with 0.25% trypsin-ethylene diamino tetra-acetic acid (EDTA) solution (Invitrogen, Milan, Italy). Fresh tissues from orthotopically implanted GI-LI-N and HTLA-230 animal models were dissected with a scalpel in Iscove's Modified Dulbecco's Minimum Essential Medium (IMDM supplemented with 2% fetal calf serum, FCS) in bath ice. 10^{10} transducing units (T.U.) of a X_7 (X = any amino acid) phage library (Ph.D.™-7 Phage Display Peptide Library Kit, New England Biolabs, Ipswich, MA) was added to 5×10^5 target cells in binding medium, and incubated for 4 h at 4 °C (first round). For successive rounds, phages were first pre-adsorbed on control cells/tissues for 1 h at 4 °C and were subsequently incubated with target tissues for 2 h at 4 °C. After 5 washes in binding medium, bound phages were recovered and amplified by infection of K91Kan *Escherichia coli* bacteria in log-phase. Phage particles were purified from bacterial culture supernatants by precipitation in NaCl/poly(ethylene glycol-8000) and titrated as described [21,22]; phage DNA was extracted and sequenced following the instructions of the Ph.D.™-7 kit (New England Biolabs).

2.4. Ex-vivo validation of phage clones targeting neuroblastoma tissues

Fifteen phage clones, chosen on the basis of the presence of repeated tripeptide motifs, were validated for their binding to NB tissues both from patients with stage IV NB and mice orthotopically implanted with GI-LI-N cells. Tumors, frozen in optimum cutting temperature (OCT) medium (Miles Chemical Co., Elkhart, IN), were cut in 5 μm-sections, fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, and histologically evaluated by staining with Mallory Trichrome (Bio-Optica, Milan, Italy). For the phage overlay binding assay, sections were washed twice in phosphate buffered saline (PBS), saturated with Protein Block Serum-Free (DAKO, Milan, Italy), and overlayed for 1 h at 4 °C with 10^8 T.U./μL of each phage clone. After extensive washing, phages were revealed by staining with a rabbit anti-fd bacteriophage antibody (1:100, Sigma, St. Louis, MO) and DcEnVision + System HRP (DAKO, Milan Italy) as substrate. Non-epithelial tumor components were identified by staining with rabbit polyclonal anti-Factor VIII (1:100, DAKO), rat monoclonal anti-CD31 (1:100, BD Biosciences, Franklin Lakes, NJ), Cy3-conjugated anti-α-smooth muscle actin mouse monoclonal (SMA-Cy3, 1:200, Sigma), and rabbit polyclonal anti-collagen I (1:200, AbCam, Cambridge, UK). For immunofluorescence staining and confocal analyses (TCS SP2 confocal microscope, Leica Microsystems, Mannheim, Germany), AlexaFluor® secondary antibodies (Invitrogen) were used.

2.5. Synthesis and in vitro validation of the NB-targeting peptides

To reproduce the original molecular environment of their phage-displayed counterpart, selected NB-targeting peptides were synthesized with the addition of the YSHS and GGG sequences at their N- and C-terminal, respectively. To favor the accessibility of liposome-bound peptides and to allow their coupling to maleimido groups (see below), an additional Cys residue was inserted at their C-terminal, resulting in the following peptide sequences (the NB-binding motifs are underlined): #1: YSHSYEGLISRGGGC; #5: YSHSHSYWLRSGGGC; #8: YSHSWSWPRELGGGC; #10: YSHSALAAHKLGGGC; #14: YSHS KSFFLSHGGGC. In some experiments, the scrambled (SCR) peptide YSHSLAKALHAGGGC was used as a control. For the cellular association assays, biotin-conjugated NB-targeting peptides (concentration range: 20–200 μg/mL) were incubated with 1×10^6 cells for 1 h at 4 °C. Samples were washed in PBS, followed by incubation for 30 min with Cy3-labeled streptavidin (Cy™3-Streptavidin, GE Healthcare). After extensive washing, Cy3-positive cells were counted by flow cytometry,