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Enhanced anti-tumor and anti-angiogenic efficacy of a novel liposomal fenretinide on human neuroblastoma

Daniela Di Paolo [a,1], Fabio Pastorino [a,*,1], Guendalina Zuccari [b], Irene Caffa [a,2], Monica Loi [a], Danilo Marimpietri [c], Chiara Brignole [a], Patrizia Perri [a], Michele Cilli [d], Beatrice Nico [e], Domenico Ribatti [e], Vito Pistoia [c], Mirco Ponzoni [a,3], Gabriella Pagnan [a,**,3]

[a] Experimental Therapies Unit, Laboratory of Oncology, Istituto Giannina Gaslini, 16148 Genoa, Italy
[b] Dept. for Life Quality Studies, Alma Mater Studiorum, University of Bologna, 47921 Rimini, Italy
[c] Laboratory of Oncology, Istituto Giannina Gaslini, 16148 Genoa, Italy
[d] Animal Facility, IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, 16132 Genoa, Italy
[e] Dept. of Basic Medical Sciences, Neurosciences and Sensory Organs, Section of Human Anatomy and Histology, University of Bari Medical School and National Cancer Institute "Giovanni Paolo II", 70124 Bari, Italy

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ABSTRACT

Neuroblastoma is an embryonal tumor originating from the simpatico-adrenal lineage of the neural crest. It approximately accounts for about 15% of all pediatric oncology deaths. Despite advances in multimodal therapy, metastatic neuroblastoma tumors at diagnosis remain a clinical challenge. Retinoids are a class of compounds known to induce both terminal differentiation and apoptosis/necrosis of neuroblastoma cells. Among them, fenretinide (HPR) has been considered one of the most promising anti-tumor agent but it is partially efficacious due to both poor aqueous solubility and rapid metabolism. Here, we have developed a novel HPR formulation, by which the drug was encapsulated into sterically stabilized nanoliposomes (NL[HPR]) according to the Reverse Phase Evaporation method. This procedure led to a higher structural integrity of liposomes in organic fluids for a longer period of time, in comparison with our previous liposomal formulation developed by the film method. Moreover, NL[HPR] were further coupled with NGR peptides for targeting the tumor endothelial cell marker, aminopeptidase N (NGR-NL[HPR]). Orthotopically xenografted neuroblastoma-bearing mice treated with NGR-NL[HPR] lived statistically longer than mice untreated or treated with free HPR (NGR-NL[HPR] vs both control and HPR: $P < 0.0001$). Also, NL[HPR] resulted in a statistically improved survival (NL[HPR] vs both control and HPR: $P < 0.001$) but to a less extent if compared with that obtained with NGR-NL[HPR] (NGR-NL[HPR] vs NL[HPR]: $P < 0.01$). Staining of tumor sections with antibodies specific for neuroblastoma and for either pericytes or endothelial cells evidenced that HPR reduced neuroblastoma growth through both anti-tumor and anti-angiogenic effects, mainly when delivered by NGR-NL[HPR]. Indeed, in this group of mice a marked reduction of tumor progression, of intra-tumoral vessel counts and VEGF expression, together with a marked down-modulation of matrix metalloproteinases MMP2 and MMP9, was observed. In conclusion, the use of this novel targeted delivery system for the apoptotic and antiangiogenic drug, fenretinide, could be considered as an adjuvant tool in the future treatment of neuroblastoma patients.

1. Introduction

Neuroblastoma (NB) arises along the sympathetic nervous system, localizing in the abdomen, and preferentially in the adrenal gland. NB is considered the most frequent extra-cranial solid tumor of infancy [1] and accounts for approximately 15% of all pediatric oncology deaths. While the overall survival rate for children with low- and intermediate-risk NB has been consistently improved, less than 40% of high-risk NB patients survive, in spite of the intensification of the multi-agent induction therapy, along with surgery [2]. Consequently, further advances in therapy are necessary to target NB tumor cells in a more and efficient way to gain clinical benefits without substantially increasing toxicity [3]. Due to the success of 13-*cis*-retinoic acid in high-risk patients with elevated frequency of relapse from minimal residual disease [4], an increased scientific interest has been consolidated in developing retinoids, a known class of molecules able to trigger both terminal differentiation and apoptosis/necrosis of NB cells [5,6]. In this *scenario*, newer chemotherapy approaches also rely on the addition of

* Corresponding author. Tel.: +39 010 5636342; fax: +39 010 3779820.
** Correspondence to: G. Pagnan, Laboratory of Oncology, Istituto Giannina Gaslini, 16148 Genoa, Italy. Tel.: +39 010 5636342; fax: +39 010 3779820.
E-mail addresses: fabiopastorino@ospedale-gaslini.ge.it (F. Pastorino), gabriellapagnan@ospedale-gaslini.ge.it (G. Pagnan).
[1] Contributed equally to the work and should be considered joint first authors.
[2] Present address: Dept. of Internal Medicine, University of Genoa, Genoa, Italy.
[3] Contributed equally to the work and should be considered joint last authors.

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more potent retinoids, such as fenretinide (HPR), a synthetic retinoic acid derivative, which has a very low degree of toxicity relative to others and has shown efficacy as a highly active and promising therapeutic and chemopreventive agent in different experimental models and clinical trials [3,7]. However, despite good tolerability in humans, therapeutic efficacy of HPR is limited by its relatively poor bioavailability particularly from ingested tablets [8]. Indeed, the phase II study of oral capsular HPR has recently underlined how this formulation is characterized by intra-individual and interindividual variation in pharmacokinetic features as HPR is too lipophilic to easily pass the intestinal membrane [9]. For this reason, we have previously set up a liposomal formulation for delivering HPR to NB tumors without obtaining clinical relevant results [10]. Moreover, this hindrance has prompted scientists to design clinical protocols based on more appropriate HPR formulations with improved biodistribution after both oral route and intravenous injection and suitable also for pediatric use. Indeed, Maurer BJ et al. [11] have proposed a novel lipid complex to deliver HPR, called 4-HPR/Lym-X-Sorb (LXS), that was able to improve the retinoid solubility and the oral bioavailability and to significantly increase plasma and tissue levels in mice. Indeed, promising results seem to derive from ongoing new approaches to NB therapy consortium trial, according to which patients with recurrent or resistant NB are either treated with HPR orally formulated in LXS lipid matrix or as an intravenous emulsion [12]. More recently, an *in vitro* study has proposed, as novel carriers for HPR, specific amphiphilic macromolecules formed by branched polyethylene glycol covalently linked with alkyl hydrocarbon chains: in this formulation, HPR is entrapped onto hydrophobic inner cores and the resultant complexes have dimensions suitable for intravenous administration [13].

In this study, we have designed a novel nanoliposomal formulation to specifically deliver HPR to angiogenic vessels within solid tumors, such as NB. Because high vascular index in NB correlates with poor prognosis [14], it appears evident that a chemotherapy based on tumor blood vessels destruction could potentiate the direct tumor cell killing via drug release into the tumor interstitial space obtained by using selective carriers. In order to optimize our previous liposomal HPR formulation [15] and since the methods used to prepare liposomes have significant impact in the physicochemical characteristics of the agent entrapped within them, we have here employed a different loading method, known as Reverse Phase Evaporation method. Moreover, to improve the intrinsic targeting properties of the lipidic vesicles, the next step has been to arm our nanoparticles with a NGR motif-containing peptide, able to recognize a specific isoform of aminopeptidase N (APN) (CD13)-positive tumor vasculature [16,17]. Discovered by *in vivo* screening with phage libraries, this CD13 isoform is a membrane protease expressed, in tumor tissues, by endothelial cells and pericytes, and sometimes by tumor cells themselves, but it is only minimally expressed on endothelium of normal blood vessels [18]. Besides, CD13 plays a pivotal role in cancer angiogenesis, invasion and metastasis [19]. Moreover, peptides containing the NGR sequence, such as cyclic CNGRC and linear GNGRG motives, have been successfully employed by us and other researchers for delivering different antitumor agents to tumor blood vessels, including doxorubicin [18,20,21].

Therefore, in the present study, we have developed novel sterically stabilized nanoliposomes decorated with NGR-motif peptides to enhance the anti-tumor activity of HPR on NB *in vivo*. Compared to the untargeted formulation, these vascular-targeted liposomes were more effective in triggering apoptosis of tumor cells, in reducing the number of tumor vessels, and finally in inducing a statistically significant increased mice lifespan. The relevance of our novel liposomal nanomedicine could be in providing a more specific tool for adjuvant therapy of neuroblastoma.

2. Materials and methods

2.1. Chemicals

All reagents of biochemical and molecular grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogenated soy phosphatidylcholine (HSPC), cholesterol (CHE), 1,2-distearoyl-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG_{2000}), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] modified with a maleimido group at the distal terminus chain (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethyleneglycol)-2000], DSPE-PEG_{2000}-MAL), used for liposomes preparation, were from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesteryl-[1,2-^{3}[H]-(*N*)]hexadecyl ether (^{3}[H]CHE) was obtained from Perkin-Elmer Italia S.p.A. (Monza, Italy).

Fenretinide (*N*-4-hydroxyphenyl retinamide, HPR) was kindly provided by Dompè (L'Aquila, Italy) as lyophilized yellow powder. For loading into the liposomes, HPR were dissolved at 0.1 M in chloroform and methanol (1:1 molar ratio) and stored at −20°C until use. For *in vivo* experiments, free HPR was prepared as described [22].

2.2. Cell line and culture conditions

The human neuroblastoma (NB) cell line, GI-LI-N, was grown in Dulbecco's modified medium (Sigma), as previously reported [23]. Moreover, GI-LI-N cells were tested for mycoplasma contamination, cell proliferation, morphology evaluation, and multiplex short tandem repeat profiling test, both after thawing and within six passages in culture.

2.3. Characterization of liposomes

2.3.1. Liposomes preparative methods and HPR loading

Stealth liposomes (SL) were synthetized as reported [15] while the novel liposomal formulation, named as stealth nanoliposomes (NL), were prepared according to previous methods, with slight modifications [15,24,25]. Briefly, non-targeted NL and vascular-targeted NL (NGR-NL) were synthetized from HSPC:CHE:DSPE-PEG_{2000}, 2:1:0.1 molar ratio, and HSPC:CHE:DSPE-PEG_{2000}:DSPE-PEG_{2000}-MAL, 2:1:0.08:0.02 molar ratio, respectively. In some preparations, ^{3}[H]CHE was added as a nonexchangeable, nonmetabolizable lipid tracer. Lipids were dissolved in chloroform at 10 mM. Then, lipids and HPR were combined at the molar ratio of 11:1. Subsequently, distilled water was added, and the mixture was vortex, emulsified by sonication for 4 min (200 W) at 4 °C using a probe sonicator (Sonicator-ultrasonic liquid processor XL, Misonix Incorporated, Farmingdale, NY, USA) and then processed by reverse-phase evaporation by means of a rota-evaporator (Laborota 4000 Heidolph, Asynt, Isleham, Cambridgeshire, UK). Following hydration in distilled water, liposomes were extruded (LiposoFast-basic extruder, Avestin Inc., Ottawa, Canada) through a series of polycarbonate filters of pore size ranging from 0.4 μm down to 0.1 μm and the external buffer was exchanged by passing the liposomes through a Sephadex G-50 column in HEPES buffer (25 mM HEPES, 140 mM NaCl, pH 7.4). HPR concentration was evaluated by absorbance at 340 nm (Infinite M200 Monochromator Instrument, Tecan Italia, Milan, Italy).

2.3.2. Stability of liposome formulations

Leakage of HPR from liposomes was measured by dialysis in HEPES buffer at 4 °C, sampling the contents of the dialysis bag (M.W. cutoff: 100 kDa) at increasing time intervals and determining the absorbance, as above. The same procedure was also carried out in 25% human plasma from healthy donors in HEPES buffer at 37 °C.

2.3.3. Coupling of NGR peptide to nanoliposomes

NGR peptide was coupled to the external surface of NL in order to increase its accessibility, as previously described [26].

2.3.4. Light scattering experiments

Particle size (in nm), polydispersity index (PdI) and zeta potential (*Z*-potential in mV) of liposomal preparations were measured at 25 °C using a Malvern Nano ZS90 light scattering apparatus (Malvern Instruments Ltd., Worcestershire, UK), at a scattering angle of 90°.