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

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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, amionopeptidase 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 metalloproteinasises 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.

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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
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 isofrom 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 CGNGC and linear GNGRG motifs, 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-distearyl-glycerol-3-phosphoethanolamine-N[-methoxy(polyethylene glycol)]-2000 (DSPE-PEG2000), 1,2-distearyl-sn-glycerol-3-phosphoethanolamine-N[-methoxy(polyethylene glycol)]-2000 modified with a maleimido group at the distal terminal chain (1,2-distearyl-sn-glycerol-3-phosphoethanolamine-N[maleimide(polyethylene glycol)]-2000), DSPE-PEG2000-MAL, used for liposomes preparation, were from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesteryl-[1,2-3H]-([H]-N)hexadecyl ether ([3H]CHE) was obtained from Perkin-Elmer Italia S.p.A. (Monza, Italy). Fenretinide (N-4-hydroxyphenyl retinamide, HPR) was kindly provided by Dompé (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-PEG2000, 2:1:0.1 molar ratio, and HSPC:CHE:DSPE-PEG2000:DSPE-PEG2000-MAL, 2:1:0.08:0.02 molar ratio, respectively. In some preparations, [3H]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 1:1. Subsequently, distilled water was added, and the mixture was vortexed, 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 (Labortor 4000 Heidolph, Asyst, Islesham, Cambridgeshire, UK). Following hydration in distilled water, liposomes were extruded (LiposoFast-basic extruder, Heidolph, Asyst, Isleham, Cambridgeshire, UK) 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°.
Both SL and NL dispersions were diluted 100 times with the same buffer used for their setting to avoid multiscattering phenomena. Zeta potential values of liposomes were recorded with the same apparatus, following a 1:10 dilution in distilled water or in phosphate buffered saline (PBS) (Sigma) at 25 °C. The results from these light scattering experiments were presented as the average values obtained using samples from three different batches ± standard deviation.

2.4. In vivo therapeutic studies

All experiments involving animals were reviewed and approved by the licensing and ethical committee of the IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy, and by the Italian Ministry of Health. All in vivo experiments were done with the use of 11 mice per group and were repeated twice.

Five-week-old female athymic (nu/nu) mice, purchased from Harlan Laboratories (Harlan Italy, S.Pietro al Natisone, Italy) and housed under specific pathogen-free conditions, were anesthetized with xylazine-ketamine mix (Xilor 2%, Bio98 Srl, Milan, Italy-Imalgene 1000, Merial Italia SpA, Milan, Italy), subjected to laparotomy, and orthotopically injected with GI-LI-N cells (1.5 × 10^6 cells in 10 μL saline solution) in the capsule of the left adrenal gland, as described [22]. No mice died as a result of this treatment. Tumors were allowed to grow for 21 days [21] and then mice were randomly assigned to five groups and intravenously (i.v.) treated with 1 mg/kg of HPR either in free form or encapsulated into both NL and NGR-NL or with saline solution (control mice), twice a week, for 6 weeks, as previously described [22]. Mice were recorded daily for body weight and general physical status. Once mice showed signs of poor health (e.g.: abdominal dilatation, dehydra
tion, paraplegia, severe weight loss), they were killed by cervical dislocation with xylazine-ketamine mix, and the day of euthanasia was assumed as the day of death. Survival times were recorded as the principal criterion for determination of treatment efficacy.

2.5. Histological evaluations

Histological evaluations of tumor tissues were performed a day after the fifth week of treatment, when mice were still in good health conditions. Briefly, orthotopic tumor-bearing mice (n = 3 per group) were sacrificed, as above. Tumor masses were collected and processed for both paraffin- and optimum cutting temperature-embedding, as described [22]. Tissue sections (3 μm thick) were first examined after staining with Mayer’s H&E (Sigma) and then stained with primary antibodies against the proliferation antigen Ki-67 (mouse anti-human Ki-67, clone 2H3, Dako, Glostrup, Denmark) for immuno
tohistochemical analysis. Sections were analyzed for apoptosis and for staining with the primary antibodies as described [22].

2.6. Statistical analysis

Results are expressed as mean values with standard deviations (S.D.). The statistical significance of differential findings between experimental groups and controls was determined by ANOVA, with the Tukey’s multiple comparison test, using Graph-Pad Prism 3.0 software (GraphPad Software, Inc., El Camino Real, San Diego, CA). Findings were considered significant if two-tailed P values were <0.05. Survival curves have been constructed with the Kaplan–Meier method and compared by Peto log-rank test (Graph-Pad Prism 3.0).

3. Results and discussion

3.1. Characterization of nanoliposomal fenretinide

In order to improve both the chemical–physical parameters, indispensible for clinical translation, and the antitumor activity of our previously reported HPR-loaded liposomes, a novel HPR nanoparticulate formulation was performed according to the principle of the Reverse Phase Evaporation method [24,27]. In a first set of experiments, we obtained sterically stabilized nanoliposomes (NL) with a diameter of 145 ± 4 nm, a PdI of 0.033 ± 0.022 and with a zeta potential of −23.5 ± 0.8 mV in water (Table 1). Thus, NL were sufficiently smaller in diameter and characterized by a narrower size distribution with homogeneously negative charge when compared to the liposomes (SL) prepared by the previous film method (size: 180 ± 6 nm; PdI: 0.07 ± 0.014; Z-potential: −22.2 ± 1 mV). When loaded with HPR, both the liposomal formulations maintained these features without any significant difference, thus underlining how HPR entrapment did not alter the liposome shell. Starting from the same HPR amount, NL were able to encapsulate a 69 ± 5% of the initially added drug, that was slightly higher than that of SL (i.e. 62 ± 7%), as reported in Table 1.

However, the new formulation retained more HPR till 14 days at 4 °C, in particular, near 50% of total amount of the entrapped drug was released from SL only within 5–6 days while NL loosed the same quantity after 14 days (Fig. 1). The studies of liposome stability together with the concomitant dimensional analysis clearly afforded the further development of NL as a more suitable formulation for in vivo HPR delivery.

Next, in order to target CD13-positive endothelial cells of angiogenic tumor blood vessels, exclusively lying in solid tumor environment including NB [18,28], a NGR motif-containing peptide [26,29] was coupled to the exterior envelop of NL, obtaining NGR-targeted NL. Specifically, the linear peptide containing the NGR motif (GNGRG) instead of cyclic ones (CNGRC) was selected to escape putative disulphide bridge formation between adjacent peptides bound to the exterior surface of liposomes [30]. The NGR density was in the range of 10 ± 2 μg peptide/μmolides, resulting in an average coupling efficiency of 65% ± 5. The physical properties of NL were not affected either by NGR coupling or by HPR loading, having these nano-particles the

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\[\text{Table 1} \]

<table>
<thead>
<tr>
<th>Physico-chemical characterization of liposomal formulations (n = 3; ± S.D.).</th>
<th>(\text{Size (nm)}^a)</th>
<th>(\text{Pd}^b)</th>
<th>(\text{Z-potential (mV)}^c)</th>
<th>(\text{EE (%)}^d)</th>
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<tbody>
<tr>
<td>Film method</td>
<td></td>
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<tr>
<td>SL[empty]</td>
<td>180 ± 6</td>
<td>0.07 ± 0.014</td>
<td>−22.2 ± 1</td>
<td></td>
</tr>
<tr>
<td>SL[HPR]</td>
<td>179 ± 4</td>
<td>0.14 ± 0.02</td>
<td>−24.2 ± 1.2</td>
<td>62 ± 7</td>
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<tr>
<td>&quot;Reverse Phase&quot; method</td>
<td></td>
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<tr>
<td>NL[empty]</td>
<td>145 ± 4</td>
<td>0.033 ± 0.022</td>
<td>−23.5 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>NL[HPR]</td>
<td>142 ± 3</td>
<td>0.07 ± 0.028</td>
<td>−24.0 ± 0.9</td>
<td>69 ± 5</td>
</tr>
</tbody>
</table>

\(^a\) Size in nanometer (nm).

\(^b\) Polydispersity index (Pd).

\(^c\) Zeta potential in water, in millivolts (mV).

\(^d\) Percentage of encapsulation efficiency (EE (%)).
same parameters as the non-targeted counterpart (Fig. 2A, B). More in details, they possessed a small diameter ranging from 139 to 150 nm with a mean Pdl value less than 0.01 indicating a very good monodisperse liposomal preparation (Fig. 2B). Moreover, the negative Z-potential values in water as well as in PBS, unchanged until one week post-preparation, indicate that these liposomes warrant characteristics of a proper rate of stability, thus representing nanoparticles suited for in vivo studies (Fig. 2B). Furthermore, the HPR loading efficiency was similar for both vascular-targeted and untargeted nanoliposomes, that means about 0.1 mM HPR/mM lipids (Fig. 2C). To demonstrate that release of HPR from NL was negligible during the time, leakage experiments were conducted in 25% human plasma in HEPES buffer at 37 °C as well as in HEPES buffer at 4 °C. Only 20% of the total amount of the entrapped drug was released up to 108 h from the beginning of the observation in both of the experimental conditions, as reported in Fig. 2D.

Because the physico-chemical features of this novel delivery system are similar to those obtained in our previously published studies [24,31,32], performed with other drug and nucleic acid liposomal formulations, we selected NL as eligible HPR lipidic carrier for investigating their putative efficaciousness in vivo.

3.2. Anti-tumor efficacy of nanoliposomal fenretinide

We next investigated whether our novel APN-targeted HPR nanoliposomal formulation was able to potentiate the anti-tumor properties of HPR, (i.e. ability to trigger apoptosis of tumor cells, and to inhibit angiogenic process [22,33]). To this aim, we used a clinically relevant NB mouse model obtained by orthotopic implantation of NB cells into the adrenal gland of nude mice to mimic a highly angiogenic and aggressive mouse model obtained by orthotopic implantation of NB cells into the adrenal gland of nude mice to mimic a highly angiogenic and aggressive mouse model obtained by orthotopic implantation of NB cells into the

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**Fig. 1.** In vitro release of fenretinide (HPR) from stealth liposomes (SL[HPR]) and from stealth nanoliposomes (NL[HPR]) measured at 4 °C in HEPES buffer. Aliquots were taken at different time points for determination of drug concentration as described in M&M. The percentage of retention was defined as the drug dose at time t divided by the initial one. All data are the average of three different experiments ± S.D.

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**Fig. 2.** Chemical-physical characterization and release profiles of HPR-loaded untargeted (NL[HPR]) and targeted (NGR-NL[HPR]) nanoliposomal particles. (A) Representative particle size distribution by intensity. (B) Physico-chemical features of both empty and HPR-loaded NL, freshly prepared and one week-old stored (C). Loading efficiency and drug to lipids ratio of both NL[HPR] and NGR-NL[HPR]. (D) Leakage experiments from NL[HPR] were conducted in 25% human plasma in HEPES buffer at 37 °C and in HEPES buffer at 4 °C. All data are the average of three different experiments ± S.D.

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![Graph](image-url)
that HPR treatment is related to angiogenesis inhibition and to an increased anti-vascular response both in vitro and in vivo [33,34]. This activity was associated to a reduced tumor vessel density as well as to a down-modulation of the expression of both vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) [39]. In this view, we sought to prove whether the antiangiogenic potential of HPR could be raised especially when the drug was targeted to CD13-expressing endothelial tumor cells. As illustrated in Fig. 5, staining of cryopreserved NB-xenograft-derived sections revealed that the VEGF expression was reduced in a statistical manner when HPR was administered to mice by NL (NL[HPR] vs control: P < 0.001) and at a greater extent when these nanoparticles were tagged with NGR-motif containing peptide (NGR-NL[HPR] vs control: P < 0.001; NGR-NL[HPR] vs NL[HPR]: P < 0.05). Likewise, a statistically significant reduction of both tumor endothelial cells and pericytes was observed when the same tissue sections were stained with anti-CD31 and anti-α-SMA antibodies, respectively (CD31: NL[HPR] vs control: P < 0.01; NGR-NL[HPR] vs control: P < 0.01; NGR-NL[HPR] vs NL[HPR]: P < 0.05; α-SMA: NL[HPR] vs control: P < 0.05; NGR-NL[HPR] vs control: P < 0.01; NGR-NL[HPR] vs NL[HPR]: P < 0.05). These last findings point out how NL could potentiate the anti-angiogenic efficacy of HPR, especially when directed to tumor vessels. Finally, due to their involvement in tissue remodeling and especially to their critical role in regulation of cancer cell progression including tumor angiogenesis [40,41], we also investigated the expression of matrix metalloproteinases (MMPs) in the tumors derived from mice, both untreated and treated with the various HPR formulations. A pronounced statistically significant inhibition of both MMP-2 and MMP-9 expressions was detected following liposomal HPR administrations to the mice, with prominent effects obtained by means of the NGR-NL[HPR] preparation (NGR-NL[HPR] vs control: P < 0.001; NL[HPR] vs control: P < 0.01; NGR-NL[HPR] vs NL[HPR]: P < 0.05) (Fig. 5).

4. Conclusions

In summary, these findings underline the leading role that angiogenesis (together with genetic abnormalities and oncogenes amplification) plays in making neuroblastoma (NB) a highly malignant pediatric
neoplasia, and strengthen the consequent need to conceive novel anti-angiogenic therapies, as widely reported [38, 42]. In this context, even if the presence of cancer stem cells within tumors could affect the effectiveness of anti-angiogenic agents [43], HPR still represents one of the most promising non-toxic drug for the treatment of NB. However, clinical translation of HPR is limited in its anti-tumor activity by a poor availability and diffusion to the neoplastic tissue [19]. To try to overcome these hindrances, we designed a new HPR formulation that, thanks to the novel method of drug’s encapsulation within a sterically stabilized lipidic envelope and to the targeting of tumor vessels, has improved the ability of HPR in triggering apoptosis of NB cells, and in reducing the tumor vasculature in vivo. Thus, this nanopolosomal formulation, due to a higher stability in biological fluids, could be proposed as a novel HPR carrier in the management of high risk NB as well as of all other tumors whose main characteristic is angiogenesis.

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