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Nanoencapsulation of Fenretinide in Glucosamine Butyrate - Gelatin Matrices as a Mean to Improve its Oral Bioavailability

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Abstract

Fenretinide (4-HPR) is the most promising retinoid derivative with anticancer activity and low toxicity profile. Despite its excellent tolerability, the formulations of 4-HPR that have been clinically evaluated until now have not provided drug plasma concentrations suitable to elicit a therapeutic response. Therefore innovative formulations able to improve the drug bioavailability would be of considerable interest. In this work we describe the preparation and functional evaluation of novel nanocapsules designed to enhance fenretinide bioavailability by oral route through the use of glucosamine butyrate as bioavailability enhancer and gelatin as a matrix forming agent. The anti-tumour activity of the nanocapsules has been tested in *in vitro* and *in vivo* models either in feeding and fasting conditions. Our data indicated that after oral administration the nanocapsules provide 4-HPR plasma concentrations in the range compatible with the antitumor activity as assessed *in vitro* expecially in fasting conditions. Moreover these novel nanocapsules have proved to be effective in tumor xenografts after oral administration.

Keywords: Nanocapsules; Fenretinide; Glucosamine butyrate; Oral bioavailability enhancer; Cell cultures; *In vivo* experiments

Background

Nanoencapsulation of antitumor drugs is an effective way to increase clinical efficacy through improvement of pharmacokinetics and pharmacodynamics properties [1]. Among the pharmacokinetic properties that may be affected by nanoencapsulation, the raise of drug bioavailability represents an important factor especially in the extravascular administrations of hydrophobic drugs where absorption controls the plasmatic drug levels over time, thus influencing the final outcome of the therapeutic response. Nanoencapsulation has proved to enhance the drug bioavailability by several mechanisms which include the increase of the drug aqueous solubility [2] and the improvement of permeability at the absorption site [3] to either the nanoencapsulated drug or the free drug released from the nanodevices. The improvement of drug bioavailability, especially by oral administration has ever been regarded as a major target in pharmaceutical technology due to the importance of the oral route for drug administration in every type of therapy. Indeed, the oral route has always gained the best patient's compliance for its manageability and it is the preferred choice for long term treatments. In the antitumor therapy in particular the improvement of drug availability by the oral route may represent a key tool to achieve a successful outcome because long term treatments are usually required after the first line treatment to control the minimum residual disease and avoid recurrence. However, the oral route is often inadequate to provide drug plasma concentrations suitable for antitumor efficacy because of the scarce availability of many drugs and the variability of drug absorption in the gastrointestinal environment. Thus nanoencapsulation may provide a valuable tool to improve the bioavailability of antitumor drugs by oral administration.

Retinoids, and retinoic acid in particular, are the most used drugs in the long term antitumor treatments aimed to control the minimum residual disease. Among them fenretinide N-(4-idrossiphenyl) retinamide (4-HPR) is now gaining increasing attention due to its low toxicity profile and potent cytotoxic activity observed in preclinical models of a large variety of tumours. Unlike retinoic acid, the main mechanism of action of fenretinide is the induction of apoptosis rather than cellular differentiation. Fenretinide-triggered apoptosis in cancer cells is mediated by ROS (Radical Oxygen Species) generation, activation of ceramide signalling pathways and 12-lipoxygenase activity and appears caspase-independent. Fenretinide has also shown anti-angiogenic and anti-metastatic effects in different tumour models. For these peculiar characteristics fenretinide is currently the most studied retinoid both as chemopreventive and chemotherapic agent [3].

However, despite its excellent tolerability, the therapeutic efficacy of fenretinide is still limited because of its poor bioavailability. The insolubility of fenretinide in aqueous solution indeed limits its absorption thus preventing the achievement of plasma concentrations suitable to elicit a therapeutic response. Bioavailability studies have been conducted until now by soft gelatin capsules, available at the National Cancer Institute, containing 100 mg fenretinide in corn oil and polysorbate 80 and representing the standard formulation for clinical trials. The results of multiple and protracted administrations of fenretinide by the soft gelatin capsules showed low plasma concentrations of the drug which was always below the minimum threshold for the onset of therapeutic activity [4,5]. An alternative formulation developed to increase the fenretinide oral bioavailability was based on a lipid matrix patented as Lym-X Sorb [6]. Even in

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this case, administration of very high drug doses (1700 mg/m^2/d) provided relatively low plasma concentrations. Other fenretinide formulations based on the drug incorporation in micellar systems [7] or in poly(lactic-co-glycolic acid) (PLGA) microparticles [8] or based on fenretinide complexation with human serum albumin [9] have been also developed and evaluated in preclinical stages with the aim to increase its bioavailability.

In this work we prepared and evaluated an innovative nanoencapsulated fenretinide formulation (HPR-NC) containing glucosamine butyrate as a novel gastrointestinal availability enhancer expected to promote both drug solubilization and absorption and gelatin as a matrix forming agent [10]. Glucosamine butyrate was prepared by conjugation of glucosamine with butyric acid because straight chain, low molecular weight fatty acids and sugar moieties are both known to interact with the intestinal epithelium modulating its permeability to drugs. Moreover their combination in a single molecule, providing an amphiphilic structure due to the hydrophilicity of the sugar and the hydrophobicity of the fatty chain, is expected to improve fenretinide solubilization in the aqueous environment of the gastrointestinal tract [11].

The nanocapsules were prepared by an interfacial coprecipitation technique in the presence of increasing amounts of glucosamine butyrate. The main physico-chemical and functional characteristics of the nanocapsules were evaluated to find out the best weight ratio between glucosamine butyrate (E) and drug (D) providing the nanocapsules endowed with the highest bioavailability and therefore the possibility to attain absorption levels across the gastrointestinal wall in therapeutically relevant concentrations [12].

Methods

Drugs and chemicals

Gelatin type B, glucosamine and butyric anhydride were purchased from Sigma–Aldrich, Fenretinide (N-(4- hydroxyphenyl)retinamide) (4-HPR) was purchased from Olon S.p.A. (Italy) all the other solvents and reagents were from Sigma. Glucosamine butyrate (GlcNBu) was synthesized following a method previously described [13]. Briefly glucosamine hydrochloride (100 mmol) was added to anhydrous methanol (150 ml) and sodium methoxide (1 eq). After stirring 5 min the resulting NaCl pp was removed by filtration and butyric anhydride (110 mmol) was added to the filtrate. The reaction mixture was stirred 4h at room temperature and then cooled at 0°C overnight. The crude GlcNBu was filtered, washed with cold ethanol followed by diethyl ether and was finally freeze dried. Acetonitrile, methanol, acetic acid and all the chemicals and solvents used for the preparation of standard solutions and mobile phase for HPLC analysis, were from Sigma-Aldrich (Milan, Italy). Ultrapure water was obtained by a Milli-Q purification system (Millipore, Bedford, MA, USA).

Preparation of the nanocapsules

The nanocapsules were prepared by an interfacial coprecipitation method carried out by mixing a fenretinide solution in ethanol, containing GlcNBu, with an aqueous solution of gelatin. The fenretinide solution was obtained by dissolving 200 mg fenretinide in 3 ml ethanol at 35°C. This solution was supplemented with 100, 200, 400 or 600 mg GlcNBu (corresponding to 0.5, 1, 2, and 3 E/D ratios respectively) and subsequently mixed with 30 ml of an aqueous solution of gelatin 3% (w:v) at 40°C. Stirring was carried out 5 min in the dark to obtain nanocapsules by coprecipitation of fenretinide and gelatin at the interface of the micelles formed by GlcNBu, triggered by the ethanol extraction towards the aqueous phase. Afterward stirring was continued in an ice bath until complete solidification of the gelatin coprecipitate which occurred below 27°C. The resulting dispersion was diluted with an excess of cold water to induce sedimentation of the solid nanocapsules to the bottom of the glass beaker. After several washing with cold water the nanocapsules were lyophilized and subsequently stored in the dark at 4°C. Empty nanocapsules were prepared by the same method in the absence of fenretinide.

Drug loading and encapsulation efficiency of HPR-NC

The HPR-NC were analysed for their drug loading and encapsulation efficiency by the HPLC method reported in the HPLC conditions section. For the HPLC analysis the nanocapsules (100 mg) were dissolved in 10 ml of a water:ethanol 90:10 (v:v) mixture at 45°C and subsequently added with 20 ml ethanol at 0°C to induce precipitation of gelatin and complete solubilisation of the fenretinide content. The drug loading and encapsulation efficiency were expressed as:

$$\text{Drug loading } \% = \left(\frac{\text{weight of the drug in the nanocapsules}}{\text{weight of the nanocapsules}} \right) \times 100$$

$$\text{Encapsulation efficiency } \% = \left(\frac{\text{weight of the drug in the nanocapsules}}{\text{weight of the drug in the preparative mixture}} \right) \times 100$$

Morfology and size of HPR-NC

The morphology and size of the nanocapsules were evaluated by transmission electron microscopy (TEM). 10 µl of the HPR-NC formulation was absorbed onto formvar-carbon coated grids (200 mesh size) for 40 min and stained with 2% aqueous phosphotungstic acid solution before viewing with a Philips CM10 transmission electron microscope at 80 kV (FEI Company, Eindhoven, the Netherlands). The images were digitally captured by SIS Megaview III CCD camera (FEI Company, Eindhoven, the Netherlands).

The average particle size and the polydispersity index of the nanocapsules were measured by Dynamic Light Scattering (Brookhaven 90 plus particle size analyzer) at 25°C. The samples for analysis were prepared by dispersion of 100 mg of nanocapsules in 10 ml of purified water. Each measure was repeated in triplicate.

Drug release from the nanocapsules *in vitro*

To evaluate the drug release from the nanocapsules the dialysis membrane method was used. 100 mg of HPR-NC were placed in a donor compartment containing 2 ml of phosphate buffer solution (PBS, pH 7.4), separated by a dialysis membrane (MWcoff 3.5 kDa) from a receiving compartment containing 100 ml PBS and 15% ethanol v/v. The apparatus was maintained at 37°C ± 0.5°C and at appropriate time points, 1 mL of the receiving medium was taken for measurement of fenretinide by HPLC. The same volume of fresh medium was added. The release experiments were performed in triplicate.

In vitro cytotoxicity of HPR-NC

Cells were plated in 96-well tissue culture plates at a density of 1x10^{3}cells/well, allowed to attach 24 h, and then left untreated or treated with growth medium containing different concentrations of 4-HPR both pure or as HPR-NC. To test the pure 4-HPR a previous dissolution in ethanol had been done followed by dilution with the culture medium at a final ethanol concentration not exceeding 0.1%. After different time periods the cytotoxic effect of fenretinide was determined by a MTT assay. To assess the biocompatibility of GlcNBu and gelatin cytotoxicity tests were also carried out in the presence of void nanocapsules. SK-N-AS and HTLA neuroblastoma cell lines

were used in the present study. They were purchased from American Type Culture Collection (ATCC) and were cultured in RPMI 1640 medium supplemented with L-glutamine 2%, 10% FBS and Penicillin/Streptomycin.

In vivo absorption studies

The absorption studies were conducted in comparison with the standard formulation available from NCI consisting in soft gelatin capsules containing 100 mg fenretinide in corn oil and polysorbate 80, in accordance with previous studies reported in the literature evaluating the absorption of fenretinide from oral formulations [14] .Athymic nude mice were gavaged with fenretinide by using the HPR-NC formulation or the reconstructed content of the soft gelatine capsules at the dose of 100 mg/kg (n=5) or 200 mg/kg (n=5) once a day. The administration was carried out for 1 day or 5 days. Four hours after the last dose the animals were sacrificed and blood was collected from a cardiac puncture. The absorbtion studies were also carried out in the presence of a tumor mass. In this case the mice were inoculated with SK-S-AN neuroblastoma cell lines (5×10^6 tumor cells/mouse) and when the tumors reached a mean volume of 300 mm^3 they were gavaged with fenretinide by the HPR-NC formulation at the dose of 100 mg/kg (n=5).The administrations were carried out 1 day or 5 days either in feeding or fasting conditions. At the end of the experiment, 4 h after the last dose, animals were sacrificed and bloodand tumors were collected. Fenretinide was quantified in plasma, and tumors using HPLC. Plasma samples were prepared for HPLC analysis by adding ice-cold acetonitrile (1:1 v:v) mixing by vortex and placing in an ultrasonic bath for 10 min. The mixture was then centrifuged at 10,000 × g at 4°C for 5 min. The supernatant was injected directly into the HPLC system. The tumors were homogenized using a tissue homogenizer with a 1:3 w:v ratio of physiologic saline (0.9% NaCl, w/v) and subsequently extracted with ice-cold acetonitrile (1:1 v:v) mixing by vortex and placing in an ultrasonic bath for 10min. The mixture was then centrifuged at 10,000 × g at 4°C for 5 min and the supernatant injected directly into the HPLC system.

In vivo efficacy

Female athymic nude mice, 6-8 weeks old, weighing 20-25 g were subcutaneously implanted with SK-S-AN neuroblastoma cell lines (5×10^6 tumor cells/mouse). The mice were then randomized into groups of six animals for the different doses of drug. When the tumors reached a mean volume of 150 mm^3 the animals were gavaged with HPR-NC at doses of 25, 50, 100 or 200 mg/kg, 3 days /week for a total of 4 weeks. As a comparison the mice were also gavaged with 200 mg/kg of the pure drug by using the reconstructed content of the NCI capsules. The experiment was carried out also in fasting conditions. In this case the animals were restricted from food access 4 hours before and after gavage. Tumor size was measured using digital calipers on alternate days and tumor volume was calculated using the following formula: $1/2\times length\times width^2$.

HPLC conditions

Chromatographic analyses were carried out on a HPLC-UV DAD model 1050 system by Agilent Technologies (Waldbronn, Germany). The separations were performed on a Luna C18 column (3 µm, 150 mm x 4.6 mm I.D.) with a Luna C18 guard column by Phenomenex (Torrance, CA, USA). Isocratic elution with a mixture of acetonitrile:water:glacial acetic acid (80:18:2, v/v/v) was employed at a flow rate of 1.0 mL/min, at room temperature, according to a described and validated method [15]. Manual injections were performed by means of a Rheodyne Model 7125 injector withµL a 200 sample loop. The eluent was monitored at the detector wavelength of 340 nm. Fenretinide stock solution was prepared at the concentration of 500 µg/mL in acetonitrile:water (70:30, v/v). Calibration curves were obtained by properly diluting the stock solution to obtain six concentration levels ranging within 0.005-1.50 µg/mL. The standard samples were then injected in triplicate and the mean peaks area (y) were plotted against the corresponding concentration (C, µg/mL) to obtain, by linear regression analysis, equations such as:

$y = 1275\ (\pm\ 11.92)\ C - 1.65\ (\pm\ 4.27);\ r^2 = 0.999,\ n = 3.$

Quantitation of fenretinide in real samples was achieved by interpolating the peak area responses to the obtained calibration graphs. The method precision was found to be in agreement with that previously reported [15]; in particular at the concentration level corresponding to the lowest calibration point the RSD% was found to be ≤ 12%, n=3. The sensitivity of the method was estimated by progressive dilution of standard solution till signal-to-noise responses of 3:1 (LOD) and 10:1 (LOQ) were obtained; it was found that LOQ corresponded to 5 ng/mL, whereas LOD was found to be 2 ng/mL.

Ethics Statement

The animal experiments were carried out in the Department of Pharmacology of the University of Bologna with the approval of the local ethics committee (Veterinary Service of the University of Bologna,) and in the Department of Therapeutic Research and Medicines Evaluation (ISS Rome). All efforts were made to minimise animal suffering and the number of animals used was kept to a minimum by the experimental design. All the procedures followed in this work were in compliance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Ethics Committee of the University of Bologna (Prot. ARIC n. 4783–X/10).

Statistical analysis

The results were presented as the mean of at least two separate experiments. The differences between values observed after the various treatments were analysed using the Student's t-test for unpaired observations. A P value < 0.05 was considered significant. The statistical significance of the in vivo experiments was analysed via an unpaired two-tailed Student t-test assuming equal variance.

Results

Characterization of HPR-NC nanocapsules

The nanocapsules prepared by the different E/D ratios were always characterized by round shapes and smooth regular surfaces as revealed by TEM analysis (Figure 1). The average size and size distribution were determined by DLS measurements. The E/D increase decreased the

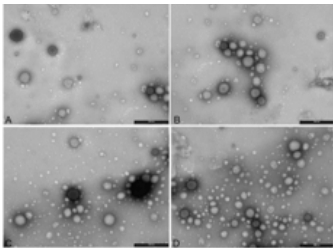

Figure 1: Representative TEM images of HPR-NC nanocapsules prepared by different Enhancer/Drug (E/D) weight ratios. A: E/D=0.5; B: E/D=1; C: E/D=2; D: E/D=3. (In all the images bar: 1000nm).

average size of the nanocapsules and also narrowed the polydispersity index indicating an unimodal size distribution at the highest E/D ratio (Table 1). Drug loading and encapsulation efficiency also increased with the E/D ratio increase (Table 1).

In vitro drug release

Fenretinide release from the nanocapsules in PBS was characterized by a lag time lasting 1-4 h followed by an enhanced rate release profile up to 10-12 h and a plateau (Figure 2). The cumulative release ranged from a minimum of 52.7% to a maximum of 68.1% after 6h and from 73.0% to 90.5% after 12 h. At each time point the most rapid release was observed from the nanocapsules prepared by the highest E/D ratio. Decreasing the E/D ratio also decreased the release rate.

In vitro cytotoxicity

In vitro cytotoxicity assays indicated that nanoencapsulation improved the in vitro activity of fenretinide with respect to the pure drug. The nanocapsules prepared with E/D=3 ratio showed the highest cytotoxic activity among the systems analysed (Figure 3). The increase in the exposure time from 24 to 72 h further improved the cytotoxicity of the nanoencapsulated fenretinide with respect to the pure drug and the effect was particularly enhanced in the presence of the E/D = 3 ratio (Figure 3). The void nanocapsules, tested at the same concentrations as the loaded ones did not provide any cytotoxicity neither at 24 nor at 72 h.

Based on both the cytotoxicity studies and the characterization studies the nanocapsules prepared by E/D =3 ratio appeared the best among the systems analysed. Therefore they were selected for the further in-vivo evaluations and have been referred as HPR-NC_3 throughout the text.

In vivo absorption

The concentration of fenretinide in plasma increased with the dose and was higher after 5 days of treatment (Figure 4). HPR-NC_3 provided higher plasma concentration of fenretinide than the soft gelatin capsules both after 1 day and 5 days (Figure 4).

In the presence of fasting conditions the plasma concentration of fenretinide was not significantly improved neither after 1 day nor after 5 days (Figure 5). On the contrary a strong accumulation of the drug in the tumor is obtained in fasting conditions after 5 days (Figure 5).

In vivo efficacy

HPR-NC_3 provided dose-dependent antitumor activity with a significant reduction of the tumor growth at 200 mg/kg (Figure 6). The same dose administered as the reconstructed content of the soft gelatin capsules provided a very limited antitumor activity, much lower than that obtained by the HPR-NC_3 formulation. Fasting conditions strongly improved the antitumor activity of fenretinide from HPR-

E/D	Particle size (nm)	Polydispersity index	Drug leading %	Encapsulation efficiency %
0.5	335.22 ± 19.2	0.5 ± 0.01	9.38 ± 2.2	61 ± 1.0
1	267.55 ± 20.6	0.37 ± 0.04	30.05 ± 2.4	76 ± 0.8
2	240.85 ± 24.1	0.28 ± 0.02	30.74 ± 2.5	80 ± 0.6
3	213.53 ± 7.9	0.34 ± 0.01	11.66 ± 2.7	87 ± 2.1

Table 1: Characteristics of the Fenretinide loaded nanocapsules prepared by different Enhancer/Drug weight ratios [E/D].

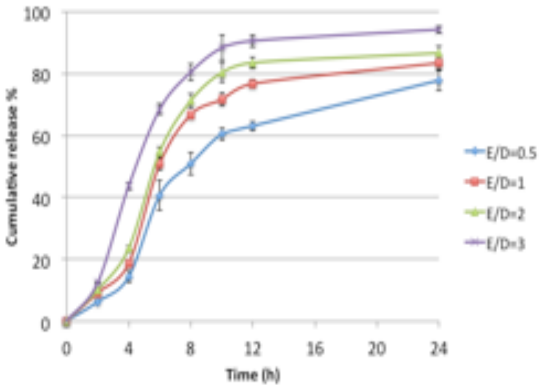

Figure 2: Release profiles of fenretinide from HPR-NC nanocapsules prepared by different Enhancer/Drug (E/D) weight ratios. Data represent mean ± SD (n=3).

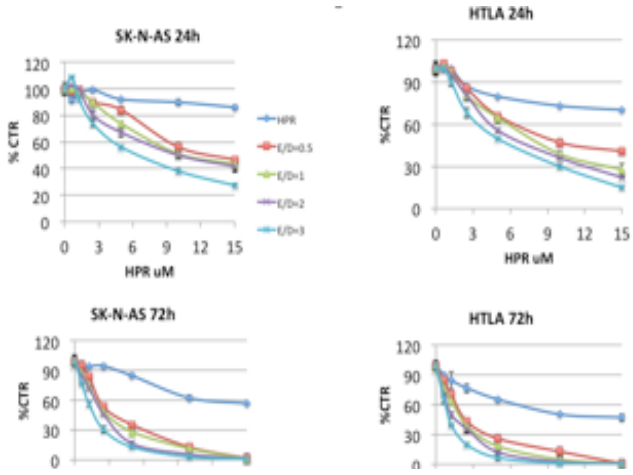

Figure 3: Cell viability of SK-N-AS and HTLA incubated with pure fenretinide (HPR) or HPR-NC nanocapsules prepared by different Enhancer/Drug (E/D) weight ratios. Data represent mean ± SD (n=3).

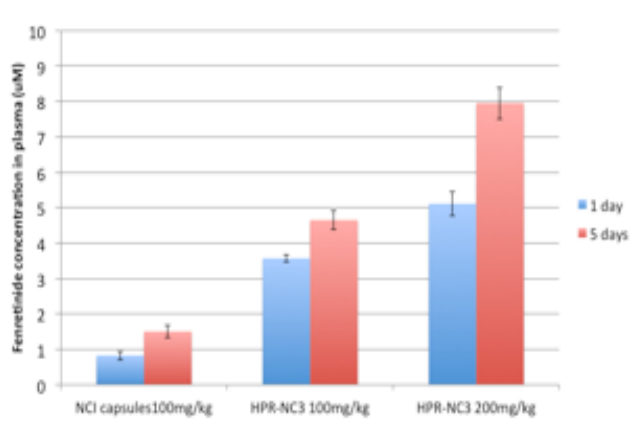

Figure 4: Fenretinide (HPR) concentrations obtained in mouse plasma after administration of HPR-NC_3 (E/D=3) nanocapsules or the reconstructed content of the NCI capsules. Athymic nude mice were orally administered at the dose of 100 mg/kg (n=5) or at the dose of 200 mg/kg (n=5) once a day. The administrations were carried out 1 day or 5 days. Animals were sacrificed 4 h after the last dose and blood was collected from a cardiac puncture.

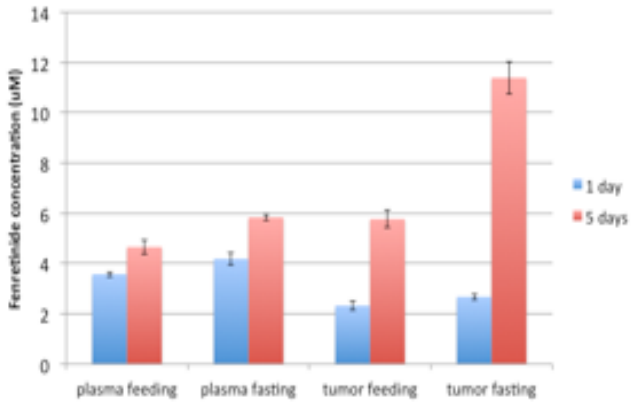

Figure 5: Fenretinide (HPR) concentrations in mice plasma and tumors after administration of HPR-NC_3 (E/D = 3) nanocapsules either in feeding or fasting conditions. The mice were inoculated with SK-S-AN neuroblastoma cell lines (5×10^6 tumor cells/mouse). When the tumors reached a mean volume of 300 mm^3 the mice were orally administered at the dose of 100 mg/kg (n=5) once a day. The administrations were carried out 1 day or 5 days either in feeding or fasting conditions. Animals were sacrificed 4 h after the last dose and blood and tumors were collected for determination of their HPR content.

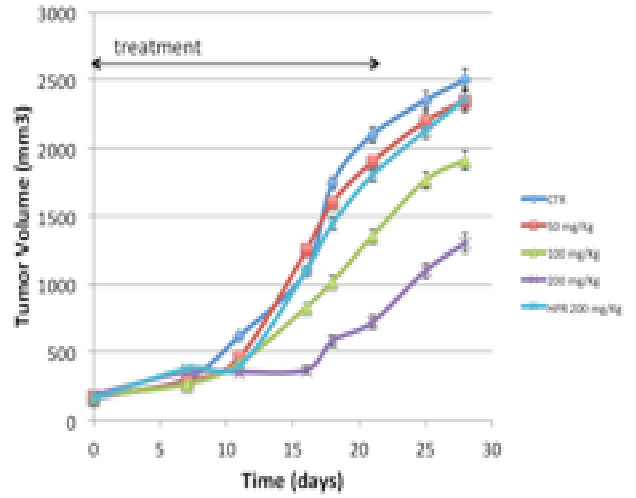

Figure 6: Tumor volume increase after administration of fenretinide. SK-S-AN neuroblastoma cell lines were established as xenografts in athymic nude mice. When the tumors reached a mean volume of 150 mm^3 the animals were gavaged with HPR-NC_3 (E/D=3) at doses of 50 mg/kg, 100 mg/kg or 200 mg/kg, 3 days /week for a total of 3 weeks. As a comparison the mice were also gavaged with 200 mg/kg fenretinide by using the content of the NCI capsules (HPR).

NC_3 indeed the administration of 100 mg/kg provided a very limited tumor growth which was significanly lower than that obtained by the same dose administered in feeding conditions (Figure 7) and was also lower than that obtained by the administration of 200 mg/kg in feeding conditions (Figures 6 and 7).

Discussion

Fenretinide has long been regarded with great interest for its limited toxicity and high antitumour activity but its low bioavailability has until now prevented its clinical use. The improvement of fenretinide bioavailability, especially by oral administration, has attracted the maximum attention as the oral route always meets the best patient's compliance and, due to its manageability, it is the preferred choice for long term treatments. The standard oral formulation of fenretinide, used until now in clinical trials and available at the National Cancer Institute, is represented by soft gelatin capsules containing 100 mg fenretinide in corn oil and polysorbate 80. Bioavailability studies, conducted after multiple and protracted administrations of these capsules, always showed plasma concentrations of fenretinide below the minimum threshold for the onset of therapeutic activity [4,5]. Alternative oral formulations have been developed to increase fenretinide bioavailability, among them a lipid matrix based formulation patented as Lym-X Sorb [6].

In this work we prepared and evaluated an innovative nanoencapsulated fenretinide formulation (HPR-NC) based on glucosamine butyrate as a novel gastrointestinal availability enhancer and gelatin as a matrix forming agent. Glucosamine butyrate was prepared by conjugation of glucosamine with butyric acid because straight chain, low molecular weight fatty acids and sugar moieties are both known to interact with the intestinal epithelium modulating its permeability to drugs [10-12]. Glucosamine butyrate is expected to improve fenretinide bioavailability by a double mechanism: solubilization and absorption. Indeed its amphiphilic nature due to the hydrophilicity of the sugar moiety and the hydrophobicity of the fatty chain may improve the fenretinide solubility in the aqueous fluids of the gastrointestinal tract. In addiction its ability to interact with the gastrointestinal epithelium may improve the drug permeability through the gastrointestinal mucosa. In our work we found that the weight ratio between glucosamine butyrate (E) and drug (D) significantly influenced the physico-chemical and functional properties of the nanocapsules. TEM analysis showed that the structure of the nanocapsules was affected by E/D, the best being obtained by the highest (E/D=3) ratio. These nanocapsules, named HPR-NC_3 were endowed with a regular spherical shape and were characterized by the lowest size and polydispersity and the highest drug loading and encapsulation efficiency among the systems analyzed. Moreover HPR-NC_3 provided the most efficient drug release. Indeed their cumulative

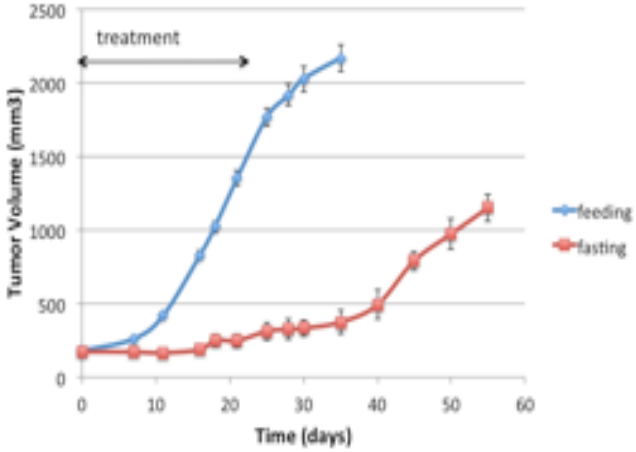

Figure 7: Tumor volume increase after administration of fenretinide. SK-S-AN neuroblastoma cell lines were established as xenografts in athymic nude mice. When the tumors reached a mean volume of 150 mm^3 the animals were gavaged with HPR-NC_3 (E/D=3) at 100 mg/kg 3 days /week for a total of 3 weeks either in feeding or fasting conditions.

drug release, 90% at 12 h, was compatible with an almost complete drug release during the average gastrointestinal transit time. Also the cytotoxicity of fenretinide towards SK-N-AS and HTLA neuroblastoma cell lines was improved in HPR-NC_3 due to the higher availability of the drug from the nanocapsules containing increased concentration of the solubility enhancer.

In comparison with the standard soft gelatin capsules the novel HPR-NC_3 nanoencapsulated formulation raised the absorption of fenretinide by oral administration both after 1 day and 5 days treatment. As a consequence also the antitumor activity of the drug from HPR-NC_3 was improved compared with the same dose administered by the content of the standard soft gelatin capsules. The administration of HPR-NC_3 in fasting conditions further improved the absorption of fenretinide from the gastrointestinal tract. Indeed higher drug concentrations were obtained both in plasma and tumors when the administration was performed in the absence of food. The increased drug absorption consequently improved the antitumor activity of fenretinide as demonstrated by the slower tumor growth obtained by the administration of 100 mg/kg in fasting conditions compared with the tumor growth observed in feeding conditions by either the same dose (100 mg/kg) or a double dose (200 mg/kg). The raise in drug absorption obtained in fasting conditions may be correlated to the ability of glucosamine butyrate to interact with the gastrointestinal mucosa modifying its physico-chemical characteristics towards enhanced permeability to drug diffusion. In the absence of food the larger accessibility of the mucosa surface may allow the establishment of additional interactions with glucosamine butyrate amplifying its enhancement effect on permeation and drug absorption. Without enhancer, on the contrary, the presence of food is well known to favour the gastrointestinal absorption of fenretinide and other retinoids [16-19]. High-fat meals, in particular, strongly increase their absorption and reduce variability. Indeed the manufacturer's summary of product characteristics recommends for retinoids to be taken with food preferably fat food like milk etc.

In conclusion the novel HPR-NC_3 formulation allows to exploit the therapeutic activity of fenretinide by oral administration. It relies on the presence of glucosamine butyrate as a solubilisation and absorption enhancer for fenretinide greatly improving its bioavailability especially in fasting conditions. HPR-NC_3 represents an effective tool for both the antitumor treatment and the control of the minimal residual disease which is often the major cause of therapy failure due to a relapse of the disease also after a first complete remission.

References

1. Brigger I, Dubernet C, Couvreur P (2002) Nanoparticles in cancer therapy and diagnosis. Adv Drug Deliv Rev 54: 631-651.
2. Pohlmann AR, Fonseca FN, Paese K, Detoni CB, Coradini K, et al. (2013) Poly(Ïµ-caprolactone) microcapsules and nanocapsules in drug delivery. Expert Opin Drug Deliv 10: 623-638.
3. Sunkireddy P, Jha SN, Kanwar JR, Yadav SC (2013) Natural antioxidant biomolecules promises future nanomedicine based therapy for cataract. Colloids Surf B Biointerfaces 112: 554-562.
4. Jasti BR, Lo Russo PM, Parchment RE (2001) Phase I clinical trial of fenretinide (NSC374551) in advanced solid tumors. Proc Am Soc Clin Oncol 20: 122.
5. Garaventa A, Luksch R, Lo Piccolo MS, Cavadini E, Montaldo PG, et al. (2003) Phase I trial and pharmacokinetics of fenretinide in children with neuroblastoma. Clin Cancer Res 9: 2032-2039.
6. Maurer BJ, Kalous O, Yesair DW, Wu X, Janeba J, et al. (2007) Improved oral delivery of N-(4-hydroxyphenyl)retinamide with a novel LYM-X-SORB organized lipid complex. Clin Cancer Res 13: 3079-3086.
7. Orienti I, Zuccari G, Falconi M, Teti G, Illingworth NA, et al. (2012) Novel micelles based on amphiphilic branched PEG as carriers for fenretinide. Nanomedicine 8: 880-890.
8. Falconi M, Focaroli S, Teti G, Salvatore V, Durante S, et al. (2014) Novel PLA microspheres with hydrophilic and bioadhesive surfaces for the controlled delivery of fenretinide. J Microencapsul 31: 41-48.
9. Durante S, Orienti I, Teti G, Salvatore V, Focaroli S, et al. (2014) Anti-tumor activity of fenretinide complexed with human serum albumin in lung cancer xenograft mouse model. Oncotarget 5: 4811-4820.
10. Whitehead K, Karr N, Mitragotri S (2008) Safe and effective permeation enhancers for oral drug delivery. Pharm Res 25: 1782-1788.
11. Whitehead K, Mitragotri S (2008) Mechanistic analysis of chemical permeation enhancers for oral drug delivery. Pharm Res 25: 1412-1419.
12. Leonard TW, Lynch J, McKenna MJ, Brayden DJ (2006) Promoting absorption of drugs in humans using medium-chain fatty acid-based solid dosage forms: GIPET. Expert Opin Drug Deliv 3: 685-692.
13. Poustie MW, Carran J, McEleney K, Dixon SJ, Anastassiades TP, et al. (2004) N-butyryl glucosamine increases matrix gene expression by chondrocytes. J Pharmacol Exp Ther 311: 610-616.
14. Villablanca JG, London WB, Naranjo A, McGrady P, Ames MM, et al. (2011) Phase II study of oral capsular 4-hydroxyphenylretinamide (4-HPR/fenretinide) in pediatric patients with refractory or recurrent neuroblastoma: a report from the Children's Oncology Group. Clin Cancer Res 17: 6858-6866.
15. Vratilova J, Frgala T, Maurer BJ, Patrick Reynolds C (2004) Liquid chromatography method for quantifying N-(4-hydroxyphenyl)retinamide and N-(4-methoxyphenyl)retinamide in tissues. J Chromatogr B Analyt Technol Biomed Life Sci 808: 125-130.
16. Doose DR, Minn FL, Stellar S, Nayak RK (1992) Effects of meals and meal composition on the bioavailability of fenretinide. J Clin Pharmacol 32: 1089-1095.
17. Colburn WA, Gibson DM, Wiens RE, Hanigan JJ (1983) Food increases the bioavailability of isotretinoin. J Clin Pharmacol 23: 534-539.
18. McNamara PJ, Jewell RC, Jensen BK, Brindley CJ (1988) Food increases the bioavailability of acitretin. J Clin Pharmacol 28: 1051-1055.
19. Schmitt-Hoffmann AH, Roos B, Sauer J, Schleimer M, Kovacs P, et al. (2011) Influence of food on the pharmacokinetics of oral alitretinoin (9-cis retinoic acid). Clin Exp Dermatol 36 Suppl 2: 18-23.