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Research Paper

Enhanced anti-neuroblastoma activity of a fenretinide complexed form after intravenous administration

Roberta Carosio[a], Vito Pistoia[a], Isabella Orienti[b,*], Franca Formelli[c], Elena Cavadini[c], Salvatore Mangraviti[d], Paolo G. Montaldo[a], Emanuela Ognio[e], Laura Emionite[e] and Guendalina Zuccari[b,*]

[a]Laboratory of Oncology, G. Gaslini Institute, Genoa, [b]Department of Pharmaceutical Sciences, University of Bologna, Bologna, [c]Chemoprevention Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, [d]Clinical Pathology Laboratory, G. Gaslini Institute and [e]Animal Model Facility, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy.

Keywords
neuroblastoma; amphiphilic dextrin derivatives; fenretinide; pharmacokinetic and therapeutic experiments

Correspondence
Guendalina Zuccari, Department of Pharmaceutical Sciences, University of Bologna, Via San Donato 19/2, 40127 Bologna Italy.
E-mail: guendalina.zuccari@unibo.it

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*Polo Scientifico-Didattico di Rimini, University of Bologna, Rimini, Italy.

Abstract

Objectives The major limitation to successful chemotherapy of neuroblastoma (NB) is the toxicity and the poor bioavailability of traditional drugs.
Methods We synthesised an amphiphilic dextrin derivative (DX-OL) able to host fenretinide (4-HPR) by complexation. In this study, we have investigated the effects of 4-HPR-loaded amphipilic dextrin (DX-OL/4-HPR) in comparison with 4-HPR alone both *in vitro* on human NB cells and *in vivo* in pseudometastatic NB models. The haemolysis assay was used as a measure of the potential damage caused by the pharmaceutical formulation *in vivo*. Pharmacokinetic experiments were performed to assess drug plasma levels in mice treated with free or complexed 4-HPR.
Key findings DX-OL/4-HPR exerted a more potent cytotoxic activity on NB cells. Complexed 4-HPR significantly increased the proportion of sub-G1 cells with respect to free 4-HPR. Dextrin derivatives showed no haemolytic activity, indicating their suitability for parenteral administration. DX-OL/4-HPR increased the lifespan and the long-term survival of treated mice over controls. The analysis of drug plasma levels indicates that the complexed drug has a higher AUC due to a reduced clearance from the blood.
Conclusions Our data suggest that DX-OL/4-HPR is an injectable formulation that is able to improve drug aqueous solubility and bioavailability.

Introduction

Neuroblastoma (NB) is the most common solid extracranial tumor in children; the disease has a wide range of clinical aggressiveness reflective of its underlying biological heterogeneity. The tumour stage, as defined by the International NB Staging System, and patient age at diagnosis are important clinical prognostic factors that strongly correlate with survival.[1] Genetic abnormalities that play a role in determining tumour phenotype and predicting outcome include amplification of the *MYCN* oncogene, chromosome 1p and 11q deletions, chromosome 17q gain and DNA polyploidy.[2] Some of these factors have been used to develop a risk-based classification scheme and categorise patients as having low, intermediate or high risk of tumour relapse in order to help guide treatment protocols.[3]

Standard therapy for NB is based on chemotherapy, surgery, treatment with ^{131}I-metaiodobenzylguanidine and autologous stem cell transplantation. Despite these aggressive therapeutic strategies, overall survival of patients suffering from high-risk NB has not increased significantly over recent years.[4,5]

Retinoids are essential regulators of cell growth, differentiation and cell death.[6] All-*trans* retinoic acid and 13-*cis* retinoic acid have been shown to induce differentiation and death in NB cells.[7] Treatment of children with advanced-stage NB with 13-*cis*-retinoic acid has increased event-free survival, but this drug shows some dose-limiting side effects.[5–7] Fenretinide (N-(4-hydroxyphenyl)retinamide; 4-HPR), a synthetic all-*trans*-retinoic acid derivative, is currently the most promising clinically tested retinoid. 4-HPR has been shown in preclinical studies to reduce the growth of breast cancer and in *in vivo* models exhibited anti-tumour activity on prostate, lung and ovarian cancers, as well as NB.[8]

The mechanism underlying the apoptosis-inducing properties of 4-HPR is not yet fully understood. It has been suggested that 4-HPR can induce apoptosis by both retinoic acid receptor dependent and reactive oxygen species dependent pathways.[9–12] Mitochondrial membrane depolarisation, which plays a key role in the process of apoptosis, has been observed in some tumour types treated with 4-HPR.[13] However, it has been reported that 4-HPR does not alter the mitochondrial membrane potential in NB.[14]

4-HPR has been evaluated in phase I–II trials in NB patients.[15,16] However, plasma levels detected in patients treated *per os* (p.o.) were lower than the drug plasma concentration required to induce apoptosis (usually 10 μM). Therefore, the pharmacological activity *in vitro* does not correspond to an equivalent efficacy *in vivo* due mainly to the poor bioavailability of oral formulation: 4-HPR itself is too hydrophobic to pass easily across the intestinal membrane. This feature, together with the hepatic first-pass effect, strongly limits the potential antitumoral efficacy of 4-HPR.[17] Thus a search for new parenteral formulations of 4-HPR able to improve drug aqueous solubility and therefore drug bioavailability is warranted. This approach would be particularly interesting in NB, in which elimination of non-proliferating tumour cells left over after chemotherapy remains a very important and unsolved challenge.[18]

Among drug delivery systems capable of improving aqueous solubility of hydrophobic drugs, polymeric micelles hold great promise. Early results have shown that 4-HPR can be successfully conjugated to poly (L-glutamic acid) or to polyvinylalcohol or encapsulated in poly(ethylene glycol)-poly(aspartic acid).[19–21] In particular, we have previously reported the synthesis of amphiphilic dextrin derivatives that are soluble in water and self-assemble in nano-aggregates endowed with hydrophobic inner cores. These derivatives can host 4-HPR by complexation (amphiphilic dextrin derivative (DX-OL)/4-HPR complex) thus raising drug aqueous solubility up to 250 mg/ml.[22] This formulation for intravenous administration of water-insoluble antitumour drugs may circumvent the incomplete gastrointestinal absorption and the hepatic first-pass effect of 4-HPR, thus increasing the drug's antitumour efficacy. In this study, we investigate the effects of this new fenretinide-loaded delivery system in comparison with 4-HPR alone both *in vitro* and in animal models of metastatic NB.

Materials and Methods

Preparation of DX-OL/4-HPR complexes

Dextrin (average molecular weight 1670, average polymerisation degree 9.33 glucose monomers) and all the other reagents and solvents employed were from Fluka Chemie GmbH (Buchs, Switzerland); fenretinide (N-(4-hydroxyphenyl)retinamide) was a gift from Dompé (Milan, Italy). As previously described,[22] dextrin 746 mg (corresponding to an average of 4.16 mmol of glucose monomers) was dissolved in N-methylpyrrolidone (NMP) and oleoyl chloride (0.50 mmol) was added in presence of polyvinylpyridine 2% cross-linked (500 mg), used as a proton scavenger. The mixture was stirred at room temperature for 24 h and an excess of diethyl ether to induce DX-OL precipitation. The solid obtained was re-precipitated twice, dialysed against water for 72 h (Spectra/Por CE Dialysis Tubing 100–500Da MWCO) and finally the solution was freeze dried. DX-OL/4-HPR solid complexes were prepared by the kneading method. DX-OL (500 mg) was dissolved in a minimum volume of NMP (2 ml) to obtain a viscous solution. 4-HPR (10 mg) was added and the viscous suspension was kneaded to homogeneity. It was subsequently diluted with water (100 ml) under stirring at room temperature until a fluid suspension was obtained. Then the suspension was dialysed against water for 72 h to remove the organic solvent and filtered (0.45 μm filter) to remove the undissolved drug. Finally the aqueous solution was freeze dried.

Tumour cells

Human NB cell lines HTLA-230, LAN-5 and IMR32 were grown in Dulbecco's modified Eagle medium (Euroclone, Milan, Italy) supplemented with 10% foetal bovine serum (Gibco, Milan, Italy) and 50 UI/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (Euroclone, Milan, Italy). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cell growth

Cells were plated in 96-well tissue culture plates, allowed to attach for 24 h, and then left untreated or treated with growth medium containing 2, 3 or 6 μM free 4-HPR (previously dissolved in ethanol); 2, 3 or 6 μM complexed 4-HPR (dissolved in PBS) or an excess (200 μM) of amphiphilic dextrin. As described previously,[23] 15 μl of 5 mg/ml 3-(4,5-diphenyltetrazolium) bromide (MTT) in PBS were then added to each well and cells were incubated for 4 h at 37°C. Formazan crystals were made soluble with DMSO (all reagents were from Sigma-Aldrich, St Louis, MO, USA). Optical densities were determined at 570 nm using a Dynatech MR5000 plate reader. Viability was expressed as a percentage of control by dividing the absorbance of each treated well by the average of the untreated or vehicle-treated controls. Results, derived from six different experiments, are expressed as mean percentage from quadruplicate wells as compared to that of control cells. IC50 was defined as the concentration of drug that decreased cell viability by 50%.

Cell cycle analysis

Cell cycle distribution was determined by flow cytometry after staining the cells with propidium iodide. Briefly, HTLA-230 cells were seeded in six-well plates and treated in the absence or presence of increasing concentrations of complexed 4-HPR (3, 6 and 10 μM) for 24 h. Cells were harvested and washed with cold PBS, centrifuged at 1200 rpm and stored overnight in 70% ethanol at −20°C. The following day, cells were washed with cold PBS, resuspended in 0.1% NP40, 100 μg/ml RNase A and 35 μg/ml propidium iodide (Sigma-Aldrich, St Louis, MO, USA), and incubated for 20 min at 37°C in the dark. The stained cells were analysed for DNA content by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA) equipped with a 15-mW argon ion laser at 448 nm and BD CellQuest Pro v.5.2 software.

Haemolysis assay

The haemolysis assay was used as a test for the interaction of compounds with the erythrocytes. Blood samples were obtained from healthy donors by venipuncture following informed consent and collected into lithium heparin-coated blood chemistry tubes and mixed gently. Blood samples (4 ml) were added with increasing doses (1.8, 3.5 and 6.0 mM) of DX-OL alone or, in one case, with 15.3 μM of complexed 4-HPR (which corresponds to the concentration reached in mouse plasma at a dose of 6 mg/kg). Incubations were carried out at 37°C with gentle tumbling of the test-tubes. After 1 h of incubation, samples were centrifuged for 5 min at 2000 × g. The results were then compared to the positive control obtained by sonication and the negative control, which contained only a mixture of blood and PBS as an indicator for the minimum haemoglobin release. In order to obtain the most sensitive measure of haemolysis, haemoglobin (Hb) concentration was determined using an ADVIA 2120 Hematology System (Siemens Medical Solutions Diagnostics, Los Angeles, USA). Haptoglobin (HPT), C reactive protein (CRP), indirect bilirubin (BilI), potassium (K^+), creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) were tested using Roche/Hitachi Cobas c Systems (Roche Diagnostics GmbH, Mannheim, Germany).

In-vivo therapeutic experiments

All experiments involving animals were reviewed and approved by the licensing and ethical committee of the National Cancer Research Institute, Genoa, Italy, and by the Italian Ministry of Health (DMS- 94/44-A). Research protocols included in this study were reviewed and approved by the Ethical Committee for Animal Experimentation (CSEA) as Animal use project 211 (responsible for the experiment execution: Dr Paolo Giuseppe Montaldo, G. Gaslini Institute Genoa), communicated to the Italian Ministry of Health having regard to the article 7 of the D. lgs. 116/92. Female CD1 nude/nude mice, 4 to 5 weeks old and weighing about 20 g were purchased from Harlan Laboratories (Udine, Italy) and housed in sterile enclosures under specific virus and antigen-free conditions. All procedures involving mice and their care were in accordance with institutional guidelines in compliance with national and international laws and policies (European Economic Community Council Directive 86/109, OJL 358, Dec. 1, 1987 and NIH Guide for the Care and Use of Laboratory Animals). Moreover, all animal experiments were in accordance with the new recommendations for cancer research community concerning the use of experimental animals in oncology.[24] CD1 *nude/nude* mice were injected i.v. with HTLA-230 (2.5×10^6/mouse, 11 mice/group) as previously described.[25] Twenty-four hours after tumour cell inoculation, mice were randomly assigned to receive 3 mg 4-HPR/kg body weight, alone (group 1) or complexed in DX-OL/4-HPR (group 2), or 6 mg 4-HPR/kg body weight complexed in DX-OL/4-HPR (group 3) or DX-OL alone 300 mg/kg (group 4), or saline solution (group 5, control mice), given slowly through the tail vein in a volume of 200 μl. The treatment was repeated three times per week for 7 weeks. To determine treatment efficacy, the animals were monitored routinely for weight loss and general behaviour, and survival time was used as the main criterion. The method of Kaplan and Meier was used to estimate survival distribution and survival time. Survival time is defined as the time in days between tumour cell inoculation and euthanasia of mice due to evidence of poor health. The statistical significance of differential survival between experimental groups of mice was determined by Kaplan–Meier curves and the log-rank (Peto) test using GraphPad Prism 3 statistical software (La Jolla, CA, USA). The experiments were repeated three times with similar results.

Pharmacokinetic experiments

Mice were injected via the tail vein (i.v.) with complexed 4-HPR or free 4-HPR 10 ml/kg, equivalent to 3 mg/kg. At selected time points (5, 10, 20, 30 min and 1, 2, 4, 24 h after the first injection, 24 h after the fourth and 24 and 72 h after the fifth), mice (three mice/group) were anaesthetised with halothane and blood was collected by retrobulbar venous plexus puncture. Blood was centrifuged at 5000 × g for 5 min at 4°C and 200 μl plasma was collected. The presence of 4-HPR, free or as a complexed drug, and of its metabolites *N*-(4-methoxyphenyl)retinamide (4-MPR)[26] and 4-oxo-*N*-(4-hydroxyphenyl)retinamide (4-oxo-4-HPR)[27] in plasma samples was evaluated by HPLC as described previously.[28] Briefly, an aliquot of 200 μl of each plasma sample was added to 400 μl of CH_3CN containing 125 μg/ml BHT, and the mixture was vortexed and centrifuged to pellet the precipitated proteins. The recovered supernatants were analysed on

a liquid chromatograph (Perkin-Elmer, Norwall, CT) fitted with a C18 (5-μm) reverse-phase column (150 × 4.6 mm) and a C18 precolumn (Perkin-Elmer, Milan, Italy). The mobile phase consisted of $CH_3CN : H_2O : CH_3COOH$ (75 : 23 : 2, vol/vol/vol) delivered at a flow rate of 2 ml/min. Detection was carried out with a Perkin-Elmer LC95 absorbance detector at 362 nm. *N*-(4-ethoxyphenyl)-retinamide (EPR) was used as internal standard. The reference standards for 4-HPR, 4-MPR and EPR were supplied by the R.W. Johnson Pharmaceutical Research Institute (Spring House, PA, USA); the reference standard for 4-oxo-4-HPR was kindly supplied by Rottapharm (Monza, Milan, Italy). For the quantitative evaluation, reference standard curves were set up with different known amounts of DX-OL/4-HPR, 4-HPR, 4-MPR and 4-oxo-4-HPR. The limits of quantification were: 0.03, 0.008, 0.012 and 0.05 μM for DX-OL/4-HPR, 4-HPR, 4-MPR and 4-oxo-4-HPR, respectively. The standard curves were linear in the concentration range of 0.008–12 μmol/l. The intra- and interassay accuracies for all the compounds were included in the 90–115% range and the intra- and interassay precisions were less than 15%. 4-HPR plasma concentration versus time data were analysed by non-compartmental pharmacokinetic methods using WinNonlin (version 2.0; Parsight, Mountain View, CA, USA) and the terminal elimination half-life ($t_{1/2}$) and the area under the concentration–time curve to infinity (AUC) were estimated.

Statistical methods

All in-vitro data derive from at least three independent experiments and results are expressed as mean values with 95% confidence intervals. The statistical significance of differential findings between experimental and control groups was determined by ANOVA with the Tukey's multiple comparison test using GraphPad Prism 3.0 software (Graph-Pad Software, Inc, San Diego, CA, USA). These findings were considered significant if two-tailed *P* values were less than 0.05.

The significance of the differences between experimental groups ($n = 11$ mice per group) in the survival experiments was determined by Kaplan–Meier curves by the use of the Peto's log-rank test in Graph-Pad Prism 3.0 software. These findings were considered significant if *P* values were less than 0.05.

Results

In-vitro effects of 4-HPR and DX-OL/4-HPR on NB cell lines

Cell viability studies carried out on the adrenergic MYCN-amplified HTLA-230, LAN-5 and IMR32 NB cell lines as the amplified expression of the N-Myc proto-oncogene have been extensively correlated with the highly malignant behaviour and poor prognosis of NB due to the high-risk of metastatic tumours.[29] In order to evaluate the in-vitro activity of 4-HPR and DX-OL/4-HPR against NB cells, MTT assay and cell-cycle analysis were performed.

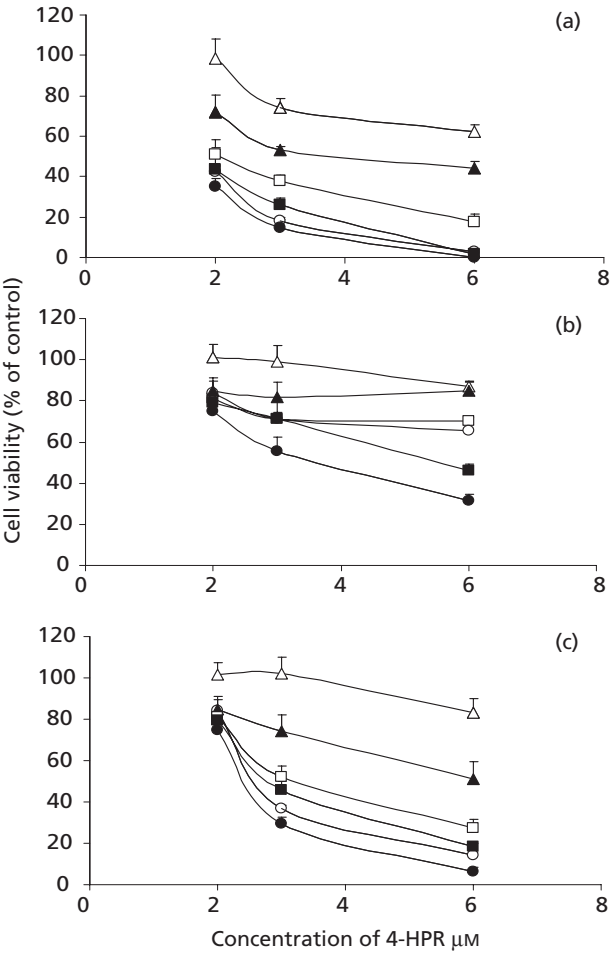

Figure 1 In-vitro activity of free 4-HPR (open points) and 4-HPR complexed in amphiphilic dextrin (filled points) against (a) HTLA-230,, (b) LAN-5 and (c) IMR 32 NB cell lines. Cell viability was measured by the MTT method at 24 h (triangle), 48 h (square) and 72 h (circle) after treatment. Results, derived from six different experiments, are expressed as mean percentage of cell viability from quadruplicate wells as compared to that of control cells. Error bars represent 95% confidence intervals.

The in-vitro antitumour activity of free 4-HPR and complexed 4-HPR was time- and dose-dependent (Figure 1). In particular, the complexed drug was always more active than the free one. A sub-G_1 peak in the cell-cycle profiles was detected in a dose-dependent manner for HTLA-230 cells treated with free or complexed 4-HPR (Figure 2). The emergence of a sub-G_1 peak indicates that cycle-arrested cells were undergoing apoptosis. Complexed 4-HPR, at concentrations of 6 and 10 μM, markedly increased the proportion of sub-G1 cells to 36 ± 0.78 and 40 ± 0.24%, respectively, while 6 and 10 μM free 4-HPR resulted in a lower increase of sub-G1 cells to 23 ± 0.32 and 25 ± 0.71%, respectively ($P < 0.01$ and

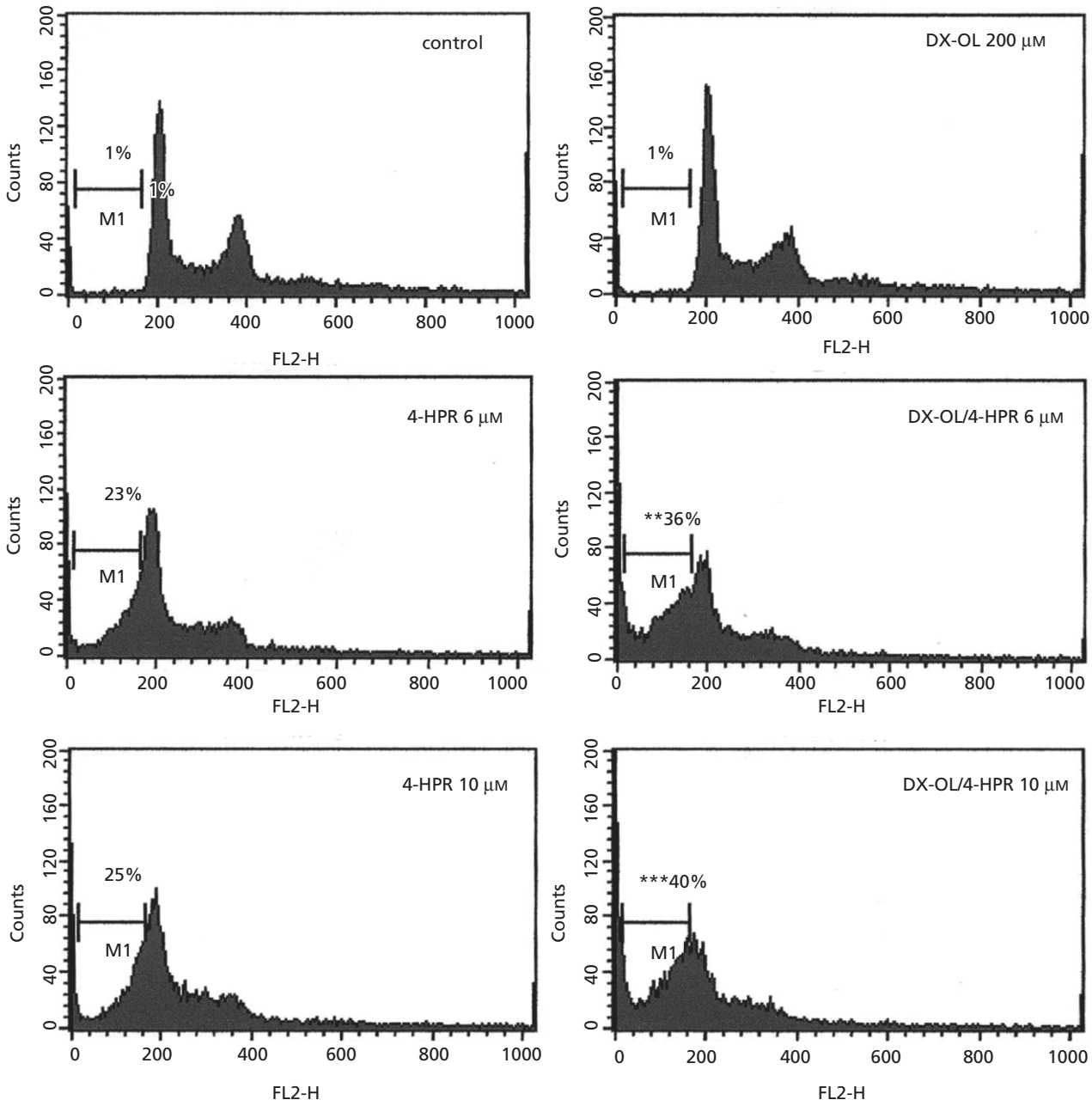

Figure 2 Representative flow cytometry analysis data of cell cycle distribution from propidium iodide staining in HTLA-230 cell line induced by free 4-HPR and complexed 4-HPR after 24 h of treatment. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus free 4-HPR compared by analysis of variance with Tukey's multiple comparison test.

$P < 0.001$ versus free 4-HPR by analysis of variance with Tukey's multiple comparison test). These results represent the mean percentage of apoptotic cells derived from six independent experiments each in triplicate. These data indicate that complexed 4-HPR is more potent than free 4-HPR at inducing apoptosis of HTLA-230 cells.

Erythrocyte haemolysis

Haemolytic activity has been proposed as an indicator of the amphiphilic dextrin toxicity *in vitro* and also as a simple and reliable measure of the membrane damage caused by the pharmaceutical formulation *in vivo*. Thus, we tested the

Table 1 Haemolitic activity of the amphiphilic dextrin DX-OL *in vitro*

Test u.m.	Hb g/dl	HPT mg/dl	RPC mg/dl	Bill mg/dl	K^+ mEq/l	CPK U/l	LDH U/l
Negative control	0.00	77.00 ± 2.15	0.46 ± 0.03	0.36 ± 0.03	4.30 ± 0.07	41.00 ± 1.12	271.00 ± 16.00
Positive control	10.90 ± 0.05	46.00 ± 1.21	0.46 ± 0.01	12.00 ± 2.09	30.00 ± 1.10	1787.00 ± 18.64	47950.00 ± 39.85
Complexed 4-HPR 15.3 μM	0.00	63.00 ± 1.37	0.46 ± 0.08	0.33 ± 0.05	3.70 ± 0.03	33.30 ± 2.97	222.00 ± 8.00
DX-OL 1.8 mM	0.00	68.00 ± 1.79	0.46 ± 0.02	0.33 ± 0.02	3.90 ± 0.04	36.60 ± 1.26	259.00 ± 17.00
DX-OL 3.5 mM	0.00	65.00 ± 2.33	0.46 ± 0.07	0.02 ± 0.01	3.60 ± 0.01	34.50 ± 2.54	219.00 ± 5.97
DX-OL 6 mM	0.00	49.00 ± 1.18	0.46 ± 0.03	0.13 ± 0.01	3.10 ± 0.07	28.10 ± 1.92	201.00 ± 6.87

Blood samples were added with increasing doses of DX-OL alone or, in one case, with 3 mg/ml of DX-OL/4-HPR (concentration reached in mouse plasma at the dose of 6 mg/kg). Haemoglobin (Hb) concentration was determined using the ADVIA 2120 Hematology System (Siemens Medical Solutions Diagnostics, Los Angeles, USA). Haptoglobin (HPT), C reactive protein (CRP), indirect bilirubin (Bill), potassium (K^+), creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) were tested using Roche/Hitachi Cobas c Systems (Roche Diagnostics GmbH, Mannheim, Germany). Each value represents the mean ± SD of five independent experiments.

haemolytic activity of the amphiphilic modified dextrin alone (patent PCT/IT2007/000552) or of DX-OL/4-HPR.

Based on the results gained from haemoglobin release from the erythrocytes, neither DX-OL/4-HPR nor DX-OL alone showed any haemolytic activity in comparison with the negative control, indicating their suitability for parenteral formulations (Table 1).

Antitumour activity of 4-HPR and DX-OL/4-HPR *in vivo*

In order to mimic the clinical features of metastatic NB, we injected nude athymic mice with the human HTLA-230 cell line (2.5×10^6/mouse, 11 mice/group) intravenously. One day after tumour cell inoculation, mice were randomly assigned to receive 3 mg of 4-HPR/kg body weight, alone (group 1) or complexed in DX-OL/4-HPR (group 2), or 6 mg of 4-HPR/kg body weight in DX-OL/4-HPR (group 3) or dextrin alone (group 4), or saline solution (group 5, control mice). The treatment was repeated three times per week for 7 weeks. Mice were daily monitored for body weight, general physical and performance status, as well as for externally visible tumour mass or ascites formation. Animals were sacrificed whenever excessive (> 25%) weight loss, huge tumour growth, massive ascites or impairment of motor functions due to spinal cord compression took place. Figure 3 shows the survival profile of treated versus control mice engrafted with HTLA-230. A highly significant increase in mean survival time was observed in mice that received DX-OL/4-HPR, both at the dose of 3 mg/kg (**$P < 0.01$, log-rank test Peto) and 6 mg/kg (***$P < 0.001$, log-rank test Peto). Moreover four out of 11 mice (36%) treated with DX-OL/4-HPR 3 mg/kg and three out of 11 mice (27%) treated with DX-OL/4-HPR 6 mg/kg were healthy and alive at the end of the experiment (150 days after tumour inoculum) and showed no evidence of macroscopic disease at necropsy. In addition, while control mice underwent rapid and extensive metastatic tumour growth, involving mainly the adrenal gland, kidney, ovary, liver, spleen and bone marrow, these events took place more slowly and less extensively in treated mice.

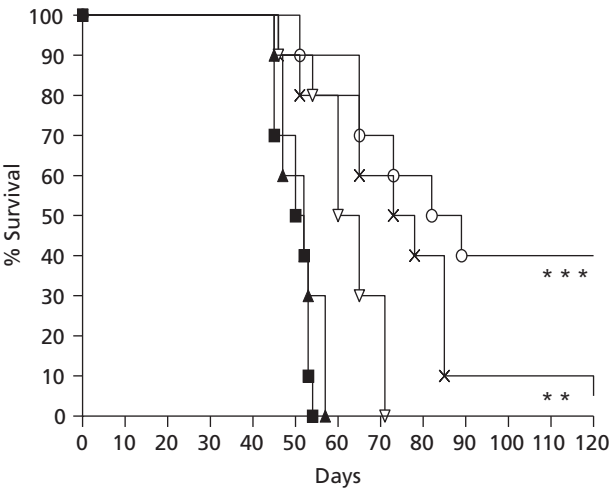

Figure 3 Survival of human neuroblastoma-bearing CD1 *nude/nude* mice after free 4-HPR or complexed 4-HPR treatment. CD1 *nude/nude* mice (5 weeks of age, female) were injected i.v. with HTLA-230 (2.5×10^6/mouse, 11 mice/group). 24 h after tumour cell inoculation, the animals received free 4-HPR (3 mg/kg, i.v. in a sterile saline solution; (open triangles)) or two doses of complexed 4-HPR (3 mg/kg, i.v.;(x) and 6 mg/kg, i.v.; (open circles)) or dextrin alone (filled triangles), or saline solution (filled squares, control mice), given in a volume of 200 μl. The dose of empty dextrin was the equivalent amount of polymer complexing 4-HPR at the higher dose. The treatment was repeated three times per week for 7 weeks. *P* values were calculated through analysis of variance with Tukey's multiple comparison test, and survival curves were compared with the use of Peto's log-rank test. Statistically significant differences compared with PBS-treated group ***P is <0.001 for 6 mg 4-HPR/kg group and **$P < 0.01$ for 3 mg 4-HPR/kg.

4-HPR pharmacokinetics

The plasma levels of 4-HPR, as a free drug or complexed, found at different intervals from single and repeated

Table 2 Plasma levels in CD1 *nude/nude* mice injected with 3 mg/kg of free or complexed 4-HPR

No. of treatments	Interval from treatment	4-HPR μmol/l	DX-OL/4-HPR μmol/l
1	5 min	10.56 ± 0.93	10.51 ± 2.70
	10 min	4.62 ± 1.16	8.78 ± 0.64
	20 min	3.25 ± 0.49	4.83 ± 1.00
	30 min	3.22 ± 0.26	5.58 ± 0.94
	1 h	1.76 ± 0.00	4.42 ± 0.46
	2 h	1.55 ± 0.20	1.77 ± 0.23
	4 h	0.82 ± 0.07	0.90 ± 0.10
	24 h	0.14 ± 0.02	0.15 ± 0.02
4	24 h	0.12 ± 0.04	0.11 ± 0.04
5	24 h	0.17 ± 0.04	0.13 ± 0.02
	72 h	nd*	nd**

Means and SD of free or complexed drug peak concentration. nd* ≤ 0.008 μM; nd** ≤ 0.03 μM.

administrations of 10 ml/kg, equivalent to 3 mg/kg, are reported in Table 2. As expected, the dose of 4-HPR administered to the two groups being the same, the plasma concentrations of 4-HPR at the earliest interval (5 min) were similar to mice treated with 4-HPR or DX-OL/4-HPR (Table 2). Then, from 10 min to 1 h, 4-HPR plasma levels were 1.5–2.5 times higher in mice receiving DX-OL/4-HPR. At all the other investigated intervals no difference in drug levels was found between the two groups. The $t_{1/2}$ for 4-HPR was similar in the two groups (6.6 and 6.8 h in mice receiving 4-HPR and DX-OL/4-HPR, respectively), whereas the AUC was higher in mice treated with DX-OL/4-HPR (22.9 vs 18.3 μMh). The levels of 4-MPR, the main 4-HPR metabolite, were similar in the two groups at all the investigated times (always < 1 μM) and 4-oxo-4HPR was not detectable (data not shown).

Discussion

The ideal immediate-release injectable formulation is obviously an aqueous isotonic solution. Unfortunately drugs are often poorly soluble in water so the first approaches to these problems have been to attempt changes in solution pH and/or the addition of a cosolvent (dimethylsulfoxide, dimethylacetamide, polyethylene glycol, propylene glycole, ethanol). If these attempts fail, the most commonly used approaches in increasing ranking of complexity are complexation, use of an organic solvent/surfactant, oil-in-water emulsions and liposomes.[30] Our previous work[22] dealt with the synthesis of a series of amphiphilic dextrins obtained by conjugation with hydrocarbon chains at a substitution degree of about 0.1 mole of hydrocarbon chain per mole of glucose monomer, as confirmed by ^{1}H-NMR. The conjugates were highly soluble in water, able to host hydrophobic drugs and dissolved with formation of nano-aggregates. Particle size analysis confirmed the dimensional suitability of the complexes for parenteral administration.

The present article expands on the biological evaluation of the most promising amphiphilic dextrin derivative obtained – the dextrin linked to an oleoyl substituent. 4-HPR complexed with DX-OL has proved to be a stable, easy-to-prepare injectable formulation that solubilises the drug at the desired concentration, provokes modest reactions at injection sites and provides an environment where the lipophilic drug has sufficient chemical stability. With such excipients, the complexed drug molecule will not precipitate upon dilution, unlike the cosolvent approach. In our study with DX-OL/4-HPR, this difference resulted in a better biological performance. The superior activity of DX-OL/4-HPR over 4-HPR in in-vitro experiments is in accordance with increased availability of the drug due to both its enhanced aqueous solubilisation in complexed form and the ability of the complex to release the free drug. Actually the complex behaves as a drug reservoir, which continuously supplies the aqueous phase with the drug and promotes drug absorption through cell membranes. The increased activity of DX-OL/4-HPR vs 4-HPR could also be related to differences in the internalisation pathways between the free and the complexed drug. The DX-OL/4-HPR complex may enter the cell by endocytosis and thus escape endo-lysosomal digestion and retention.

When the DX-OL/4-HPR complex is administered intravenously, it dissociates completely, as the major driving force for drug release is simple dilution into the systemic circulation. If we consider the parenteral injection of a small volume to a human subject, the volume of distribution (Vd) for an hydrophilic polysaccharidic carrier is said to be that of extracellular water, about 20% of total body weight (14 l for a 70 kg patient). Therefore a 5 ml injection of the drug–amphiphilic dextrin formulation would result in a 1:2800 dilution and, for most drugs, this would be sufficient to completely dissociate the drug from the dextrin.[31]

Here we show that DX-OL/4-HPR, injected i.v., is significantly more potent than free 4-HPR at inhibiting tumour growth in a metastatic model of human NB. This result, which is translationally relevant, is explained by pharmacokinetic studies. The analysis of drug plasma levels in mice

treated with 4-HPR or DX-OL/4-HPR indicates that when the drug is complexed in DX-OL it has a reduced clearance from the blood. The dose administered was in fact the same, but the AUC was higher in mice treated with DX-OL/4-HPR. In mice treated with DX-OL/4-HPR, the higher AUC of 4-HPR does not seem to be due to a lower metabolism since, as in mice treated with 4-HPR, 4-oxo-4-HPR was not detectable and the levels of 4-MPR were similar. The clearance being equal to the dose divided by the AUC, the results are thus indicative of higher drug retention in the blood due to lower clearance.

Conclusions

The present article deals with the study of a chemically modified dextrin that is soluble in water and able to self-assemble in nano-aggregates endowed with hydrophobic inner cores. This pharmaceutical formulation proved to prolong in a highly significant way mean survival (and, in a variable percentage of cases, even allow complete recovery) when injected in nude mice previously grafted with human NB cells. As more water-insoluble drugs are discovered through modern screening techniques, the need for significant formulation efforts in preclinical development will increase. DX-OL offers an additional tool for the formulator to overcome some of the formulation and delivery limitations of hydrophobic drugs. The major strengths of this approach are the potential ability to deliver safely after parenteral administration a number of intractable lipophilic drugs (an example is the use of Cremophor EL in the paclitaxel formulation[32]) and improved *in vivo* performance due to solubility enhancement.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Maris JM, Matthay KK. Molecular biology of neuroblastoma. *J Clin Oncol* 1999; 17: 2264–2279.
2. Maris JM. The biologic basis for neuroblastoma heterogeneity and risk stratification. *Curr Opin Pediatr* 2005; 17: 7–13.
3. Yu AL *et al.* Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *N Engl J Med* 2010; 363: 1324–1334.
4. Matthay KK *et al.* Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. *N Engl J Med* 1999; 341: 1165–1173.
5. Bagatell R *et al.* Outcomes of children with intermediate-risk neuroblastoma after treatment stratified by MYCN status and tumor cell ploidy. *J Clin Oncol* 2005; 23: 8819–8827.
6. Simoni D, Tolomeo M. Retinoids, apoptosis and cancer. *Curr Pharm Des* 2001; 7: 1823–1837.
7. Villablanca JG *et al.* Phase I trial of 13-cis-retinoic acid in children with neuroblastoma following bone marrow transplantation. *J Clin Oncol* 1995; 13: 894–901.
8. Sogno I *et al.* Angioprevention with fenretinide: targeting angiogenesis in prevention and therapeutic strategies. *Crit Rev Oncol Hematol* 2010; 75: 2–14.
9. Clifford JL *et al.* Retinoid receptor-dependent and -independent effects of N-(4-hydroxyphenyl)retinamide in F9 embryonal carcinoma cells. *Cancer Res* 1999; 59: 14–18.
10. Fanjul AN *et al.* 4-Hydroxyphenyl retinamide is a highly selective activator of retinoid receptors. *J Biol Chem* 1996; 271: 22441–22446.
11. Sun SY *et al.* Mediation of N-(4-hydoxyphenyl)retinamide-induced apoptosis in human cancer cells by different mechanisms. *Cancer Res* 1999; 59: 2493–2498.
12. Lovat PE *et al.* Bak: a downstream mediator of fenretinide-induced apoptosis of SH-SY5Y neuroblastoma cells. *Cancer Res* 2003; 63: 7310–7313.
13. Holmes WF *et al.* Comparison of the mechanism of induction of apoptosis in ovarian carcinoma cells by the conformationally restricted synthetic retinoids CD437 and 4-HPR. *J Cell Biochem* 2003; 89: 262–278.
14. Lovat PE *et al.* Effector mechanisms of fenretinide-induced apoptosis in neuroblastoma. *Exp Cell Res* 2000; 260: 50–60.
15. Garaventa A *et al.* Phase I trial and pharmacokinetics of fenretinide in children with neuroblastoma. *Clin Cancer Res* 2003; 9: 2032–2039.
16. Villablanca JG *et al.* Phase I trials of oral fenretinide in chidren with high-risk solid tumors: a report from the children's oncology group (CCG 09709). *J Clin Oncol* 2006; 24: 3423–3430.
17. Kokate A *et al.* Transport of a novel anti-cancer agent, fenretinide across Caco-2 monolayers. *Invest New Drugs* 2007; 25: 197–203.
18. Reynolds CP *et al.* Retinoid therapy of high-risk neuroblastoma. *Cancer Lett* 2003; 197: 185–192.
19. Zou C *et al.* Antitumor activity of 4-(N-hydroxyphenyl)retinamide conjugated with poly(L-glutamic acid) against ovarian cancer xenografts. *Gynecol Oncol* 2007; 107: 441–449.
20. Orienti I *et al.* Fenretinide-polyvinylalcohol conjugates: new

systems allowing fenretinide intravenous administration. *Biomacromolecules* 2007; 8: 3258–3262.
21. Okuda T *et al*. Enhanced in vivo antitumor efficacy of fenretinide encapsulated in polymeric micelles. *Int J Pharm* 2009; 16: 389–398.
22. Orienti I *et al*. Improvement of aqueous solubility of fenretinide and other hydrophobic anti-tumor drugs by complexation with amphiphilic dextrins. *Drug Deliv* 2009; 16: 389–398.
23. Alley MC *et al*. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* 1988; 48: 589–601.
24. Workman P *et al*. Guidelines for the welfare and use of animals in cancer research, helpful in this regard. *Br J Cancer* 2010; 102: 1555–1577.
25. Raffaghello L *et al*. In vitro and in vivo antitumor activity of liposomal Fenretinide targeted to human neuroblastoma. *Int J Cancer* 2003; 104: 559–567.
26. Formelli F *et al*. Five-year administration of fenretinide: pharmacokinetics and effects on plasma retinol concentrations. *J Clin Oncol* 1993; 11: 2036–2042.
27. Villani MG *et al*. Identification of the fenretinide metabolite 4-oxo-fenretinide present in human plasma and formed in human ovarian carcinoma cells through induction of cytochrome P450 26A1. *Clin Cancer Res* 2004; 15: 6265–6275.
28. Formelli F *et al*. Pharmacokinetics of oral fenretinide in neuroblastoma patients: indications for optimal dose and dosing schedule also with respect to the active metabolita 4-oxo-fenretinide. *Cancer Chemother Pharmacol* 2010; 66: 993–998.
29. Schwab M. MYCN in neuronal tumours. *Cancer Lett* 2004; 204: 179–187.
30. Strickley RG. Solubilizing excipients in oral and injectable formulation. *Pharm Res* 2004; 21: 201–230.
31. Stella VJ, He Q. Cyclodextrins. *Toxicol Pathol* 2008; 36: 30–42.
32. ten Tije AJ *et al*. Pharmacological effects of formulation vehicles : implications for cancer chemotherapy. *Clin Pharmacokinet* 2003; 42: 665–685.