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Nanomedicines in the future of pediatric therapy ☆

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ABSTRACT

Nanotechnology has become a key tool to overcome the main (bio)pharmaceutical drawbacks of drugs and to enable their passive or active targeting to specific cells and tissues. Pediatric therapies usually rely on the previous clinical experience in adults. However, there exists scientific evidence that drug pharmacokinetics and pharmacodynamics in children differ from those in adults. For example, the interaction of specific drugs with their target receptors undergoes changes over the maturation of the different organs and systems. A similar phenomenon is observed for toxicity and adverse effects. Thus, it is clear that the treatment of disease in children cannot be simplified to the direct adjustment of the dose to the body weight/surface. In this context, the implementation of innovative technologies (e.g., nanotechnology) in the pediatric population becomes extremely challenging. The present article overviews the different attempts to use nanotechnology to treat diseases in the pediatric population. Due to the relevance, though limited available literature on the matter, we initially describe from preliminary *in vitro* studies to preclinical and clinical trials aiming to treat pediatric infectious diseases and pediatric solid tumors by means of nanotechnology. Then, the perspectives of pediatric nanomedicine are discussed.

Contents

Abbreviations: ACTs, artemisinin combined therapies; ALN, alendronate; ANMAT, Administración Nacional de Medicamentos, Alimentos y Tecnología Médica; ARV, antiretrovirals; AS, antisense; AUC, area-under-the-curve; BMS, burning mouth syndrome; CD, cytosine deaminase; clodrolip, clodronate liposomes; CNS, central nervous system; CPT, camptothecin; DCL, drug-in-CD-in-liposome; DDS, drug delivery systems; DOTS, Directly Observed Treatment, Short Course; EC, epiphyseal cartilage; EFV, efavirenz; EMA, European Medicines Agency; EPR, enhanced permeation and retention; EuPFI, European Paediatric Formulation Initiative; EWS, Ewing sarcoma; 5-FC, 5-fluorocytosine; FDAMA, Food and Drug Administration Modernization Act; FDCs, fixed dose combinations; FP7, Seventh Framework Programme; Gal, galactose; GD2, disialogangloside; GPC, growth plate cartilage; GRiP, Global Research in Paediatrics – Network of Excellence; HER2, human epidermal growth factor receptor-2; HIV/AIDS, human immunodeficiency virus/acquired immunodeficiency syndrome; HPMA, *N*-(2-hydropropyl)methacrylamide copolymer; INH, isoniazid; i.v, intravenous; LRP, lung resistance protein; Man, mannose; MCT, medium-chain triglyceride; MDM2, murin double minute; MDR, multidrug-resistance; miRNAs, microRNAs; MRI, magnetic resonance imaging; MRP-1, multidrug resistance protein-1; MSN, mesoporous nanoparticles; MTP, muramyl tripeptide; Nano-DDS, nano-drug delivery system; NC, nanocapsule; NCAMs, neural cell adhesion molecules; NIR, near infrared; NSC, neural stem cell; PAMAM, poly(amidoamine); PDT, photodynamic therapy; PEG, poly(ethylene glycol); PEO-PPO, poly(ethylene oxide)-*b*-poly(propylene oxide) block copolymer; PIP, pediatric investigation plan; PLGA, poly(lactide-*co*-glycolide); PMs, polymeric micelles; PRD, poverty-related diseases; RGD, Arg-Gly-Asp; R&D, research and development; RIF, rifampicin; RPP, RGD-PEG-PEI; siRNA, interference RNA; TB, tuberculosis; US-FDA, US Food and Drug Administration; WHO, World Health Organization.

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1. Introduction

Nanotechnology has become a key tool to overcome fundamental (bio)pharmaceutical drawbacks of drugs such as poor aqueous solubility, low physicochemical stability and insufficient bioavailability [1–3]. Nano-drug delivery systems (nano-DDS) enable the passive or active targeting of the payload to specific cells and tissues, increasing its accumulation in the action body site and reducing adverse effects by decreasing systemic exposure to the free/active drug [4,5]. Additionally, nanotechnology has been shown to improve localized drug delivery by alternative administration routes (e.g., inhalation) in organs protected by anatomical or physiological barriers, such as the central nervous system. Irrespective of the level of complexity, the potential of nanomedicine to improve the diagnosis and the treatment of disease has been extensively documented. Thus, regardless of the regulatory demands, a reasonable number of nanomedicines have already made their way to the market [6,7]. However, all of them are for use in adults.

Because traditionally the development of pediatric treatments usually relied on the previous experience in adults, which is still scarce for most nanotechnology platforms, there do not exist approved pediatric nanomedicines yet. The application of new regulatory initiatives, such as the pediatric investigation plan (PIP) promoting specific studies in children to obtain the necessary data (efficacy and toxicity, when it is safe to do so) for the approval of a new pharmaceutical product will facilitate the authorization of medicines for children.

Children present biological and/or metabolic differences with respect to adults due to the gradual development and maturation of the different organs and systems after birth [8,9]. Thus, in the case of diseases that hit both adults and children, nanomedicines need to be primarily adjusted to fit the pediatric use, a process that might demand the development of a different pharmaceutical formulation, and then clinically trialed in children. Furthermore, in the case of diseases that are children-specific or that show substantially greater morbidity in children, nanomedicines need to be especially developed [10]. These facts regretfully enter into conflict with the complexities of the fragmented pediatric market and the challenging pediatric clinical trials that discourage researchers in both academia and industry to investigate pediatric nano-DDS.

The present article overviews the different attempts to use nanotechnology to treat diseases in children. Due to the relevance, though limited available literature on the matter, we initially describe from preliminary *in vitro* studies to preclinical and clinical trials aiming to treat pediatric infectious diseases and solid cancers by means of nanotechnology. Then, the perspectives of pediatric nanomedicine are discussed.

2. Challenges of the pediatric population

There exists global consensus among clinicians that children are not just small adults [11,12]. This statement is not a demagogic cliché but it is based on scientific evidence showing that children present differences in drug absorption, biodistribution, metabolism and excretion with respect to adults [13]. Also, derived from the differential interaction of drugs with their cellular targets, children show differences in pharmacodynamics [14–16]. In this context, it is crystal clear that the treatment of disease in children cannot be simplified to the direct adjustment of the dose to the body weight/surface [17].

An additional point of consideration is that based on differences in biology and metabolism, the pediatric subpopulation is sub-classified into subgroups, namely preterm newborn infants, term newborn infants (0–27 days), infants and toddlers (28 days-23 months), preschool children (2–5 years), school children (6–11 years) and adolescents (12–16/18 years) [9,18]. Each sub-category shows different gastrointestinal pH and transit, intestinal motility and conjugation and transport of bile salts [8,19]. Also, the level of cognitive development could impact formulation appropriateness (e.g., in the case of inhalatory products) [20] and the feasibility of clinical trials [21–23]. For instance, clinical trials in children are more complicated due to scientific, clinical, ethical, technical and logistical challenges, that have discouraged the process over the years [24–27]. Furthermore, toxicological aspects of the exposure to nanoparticles should be thoroughly assessed, especially by inhalation, because children show increased particle deposition in the lungs with respect to adults [28]. For example, the biocompatibility of several liposome formulations is well-known and a few nano-DDS have reached the clinical phase in adults [29,30]. However, information regarding their safety in children is very limited. Other platforms such as carbon nanotubes are more controversial and their clinical use seems less likely [31]. All these facts converge to reduce the flexibility and the profitability of the fragmented pediatric market and position children at the top of the vulnerability scale [32,33].

3. Infectious diseases

Despite the availability of a broad spectrum of antibiotics, the treatment of infections has become an increasing challenge of modern medicine due to the emergence of resistant pathogens. The situation is more critical in poverty-related diseases (PRDs) and even more in the case of the pediatric population owing to the reasons mentioned above. In advance the incipient progresses made at the interface of nanomedicine and the therapy of pediatric HIV, TB and malaria, three infections that claim the largest number of lives every year, will be overviewed [34].

3.1. HIV/AIDS

HIV/AIDS is the most lethal infection of our times with approximately 2.5 million annual deaths [35]. The number of antiretrovirals (ARVs) approved for pediatric administration is smaller than the one for adults and pharmacokinetic data are more limited due to the complexity of the clinical trials [36,37]. Additional drawbacks are the lack of certified liquid (e.g., solutions and suspensions), chewable [38], dispersible [39–41] and orodispersible formulations [42–44] that ease dose adjustment and ingestion and the development of pediatric fixed dose combinations (FDCs) [45,46]. Regardless of the strategy of choice, the production process should be counterbalanced to maintain medication costs under limits that ensure patient affordability [47,48]. The disease hits both the adult and the pediatric population and, thus the development of anti-HIV nanomedicines could be beneficial for all the patient sub-populations. Nevertheless, the number of research works aiming to develop nanotechnology-based anti-HIV medicines in general and for children in particular is remarkably scarce.

Nanosuspensions are dispersions of pure drug nanoparticles in a liquid pharmaceutical vehicle stabilized with surfactants [49–51]. They represent the most simplistic nano-formulation and owing to the exponential growth of the surface area with particle size reduction, a great improvement of the dissolution rate and the oral bioavailability can be achieved. Regardless of the potential of this technology to produce liquid products that would enable easier dose adjustment and swallowing, only a few ARV nano-dispersions have been explored in HIV and none of them for children [52–54].

Another promising nanotechnology used to improve the aqueous solubility of lipophilic drugs pertains to polymeric micelles (PMs), a type of self-assembly nanostructure formed by the aggregation of polymeric amphiphiles [55–57], that have been assessed by different administration routes such as parenteral [58–60], oral [61,62], intranasal [63,64] and ocular [65,66]. A number of PM-based products for the treatment of different cancers are in advanced stages of clinical evaluation [57,67]. Our group comprehensively investigated the encapsulation of the first-line ARV efavirenz (EFV) within PMs made of different types of pristine and chemically modified poly(ethylene oxide)-*b*-poly(propylene oxide) block copolymers (PEO-PPOs) towards the development of a scalable and cost-viable water-based pediatric formulation [68–73]. The solubility of the drug was increased from 4 μg/mL up to 34 mg/mL in mixed micelles, representing 8430-fold increase (Table 1) [69–73]. EFV-loaded PMs were physically stable under different storage conditions that included extreme dilution in gastrointestinal-like pH [69–73]. The oral pharmacokinetics was initially assessed in rats and compared to that of a regular extemporaneous suspension in simple syrup and an oily solution that reproduced the only commercial pediatric formulation of EFV (Sustiva® Oral Solution, 30 mg/mL) in a medium-chain triglyceride (MCT), namely Miglyol 812 [69,70]. The effect of dose, volume, post-administration dilution and micelle composition was investigated. PMs always showed a statistically significant increase of the C_{max} and the area-under-curve (AUC) with respect to the counterparts (Table 2). Importantly, the chronic administration of Miglyol 812, an approved pharmaceutical excipient, has been shown to provoke diarrhea and weight loss in an animal model [74]. One could wonder whether an excipient showing this preclinical performance would be appropriate or not for chronic pediatric administration [75,76]. The answer is probably negative and it reveals another serious constrain in pediatric pharmaceutical development: the absence of thorough toxicological information on approved pharmaceutical excipients in children. Following the promising preclinical studies, the oral pharmacokinetics of a 20 mg/mL micellar formulation (single 200 mg dose) containing only US-FDA-approved pharmaceutical excipients was preliminarily assessed in one adult healthy volunteer (protocol approved by the Ethics Committee of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Resolution No. 17193, November 8th, 2010) and compared to the performance of a 200 mg EFV reference capsule. Results were in line with those found in rats (Table 3); C_{max} and AUC increased from 1.26 μg/mL and 19.19 μg/mL/h in the capsule to 3.14 μg/mL and 28.59 μg/mL/h in the micelles. Based on these preliminary clinical data, an additional protocol aiming to extend the comparison to 12 adult healthy volunteers (200 mg single dose) has been approved by the Administración Nacional de Medicamentos, Alimentos y Tecnología Médica (ANMAT), the Argentine regulatory agency (Disposition No. 4647-12, August 9th, 2012) [77]. To the best of our knowledge, this protocol is the first pediatric nanomedicine to undergo evaluation and approval for clinical trial by ANMAT [77]. Only after the clinical evaluation in healthy volunteers, the effectiveness of this product could be assessed in infected adults and finally in infected children. An advantageous feature of this formulation is an easy production process that does not demand the use of advanced industrial equipment. This could pave the way to the scaleup. Moreover, the product could be lyophilized and supplied as a powder in individual containers that are redispersed immediately before the administration.

The central nervous system (CNS) is one of the most important HIV reservoirs [78]. High viremia could result in cognitive deficit and other developmental sequelae that are more frequent in neonates and little children [79,80]. Nanotechnology is being investigated to target ARVs to the brain by minimally invasive administration routes such as the intranasal [64]. These developments, although incipient and far from a clinical phase could represent a translatable approach to increase CNS penetration of ARVs.

Despite the potential contribution of nanotechnology to improve the therapy of pediatric HIV, there are other aspects that appear to be urgent as well. For example, EFV produces the so-called burning mouth syndrome (BMS) that could be partially overcome employing a combination of flavors, sweeteners and essences [71]. Thus, nanotechnologies need to consider other issues that are equally relevant to ensure patient compliance and the fulfillment of the therapeutic regimens.

Cyclodextrins (CDs) are oligocyclic saccharides that combine a hydrophobic cavity that can partially or totally host hydrophobic drug molecules and a hydrophilic surface that promotes dissolution in aqueous media. In this framework, drug/CD complexes have been exploited to improve the aqueous solubility and physicochemical stability of approved drugs and candidates in the pipeline [81–83]. CDs are more

Table 1
EFV apparent solubility values (S_a) in different simple and mixed polymeric micelles of pristine and *N*-methylated (Met) and *N*-allylated (Allyl) PEO-PPO copolymers. *Reproduced and adapted from Ref.[69] with permission of Future Medicine and from Ref. [70,72,73] with permission of Elsevier.*

Micelle type	Copolymer	S_a (mg/mL) (S.D.)				
		1%	3%	5%	7%	10%
Simple	F68	–	0.02 (0.02)	0.06 (0.00)	0.09 (0.02)	0.64 (0.07)
	F108	–	2.09 (0.26)	3.56 (0.43)	5.61 (0.35)	9.06 (1.08)
	F127	1.70 (0.02)	5.30 (0.13)	8.89 (0.32)	12.80 (0.82)	21.46 (0.46)
	T1107	0.25 (0.04)	2.27 (0.03)	4.80 (0.17)	8.00 (0.14)	19.48 (1.37)
	T1307	0.68 (0.09)	3.64 (0.28)	6.16 (0.07)	9.39 (0.07)	19.00 (2.34)
	T304	–	–	–	–	0.41 (0.03)
	T904	–	–	–	–	31.72 (2.97)
	Met-T904	–	–	–	–	28.68 (2.14)
	Met-T908	–	–	–	–	0.67 (0.02)
	Met-T1107	–	–	–	–	5.54 (0.43)
	Allyl-T1107	–	–	–	–	8.14 (0.33)
	Met-T1307	–	–	–	–	12.62 (0.20)
Mixed[a]	F127:T304 (75:25)	–	–	–	–	16.40 (0.82)
	F127:T304 (50:50)	–	–	–	–	10.87 (0.46)
	F127:T304 (25:75)	–	–	–	–	7.27 (0.92)
	F127:T904 (75:25)	–	–	–	–	26.16 (4.28)
	F127:T904 (50:50)	–	–	–	–	31.28 (2.35)
	F127:T904 (25:75)	–	–	–	–	33.73 (1.49)

[a] The ratio between brackets is expressed in w/w and the final polymer concentration is always 10% w/w.

Table 2
EFV oral pharmacokinetics upon the administration of 20, 40 and 80 mg/kg to Wistar rats (n = 8). The EFV concentration in all the prepared formulations was 20 mg/mL. Pluronic® F127 micellles (pF127). *Reproduced and adapted from Ref. [69] with permission of Future Medicine and from Ref. [70] with permission of Elsevier.*

Dose (mg/kg)	PK parameter	Pluronic® F127 polymeric micelles[a]		Suspension[b]			MCT solution[c]		
		Media	CV%	Media	CV%	p	Media	CV%	p
20	C_{max} (ng/mL)	1145[d]	42.7	515	37.6	<0.01	687	29.8	<0.05
	AUC_{0-24} (μg/mL/h)	7.22[d]	32.1	3.53	33.8	<0.01	4.79	36.5	<0.05
	F_r (%)	204.5	ND	100.0	ND	ND	135.7	ND	ND
40	C_{max} (ng/mL)[d]	2863[d]	28.3	1526	42.2	<0.005	1789	61.4	<0.05
	AUC_{0-24} (μg/mL/h)[d]	23.5[d]	37.1	15.1	55.2	<0.05	12.6	49.0	<0.05
	F_r (%)	155.6	ND	100.0	ND	ND	83.4	ND	ND
80	C_{max} (ng/mL)	7056[d]	21.3	3361	52.8	<0.001	2657	20.7	<0.001
	AUC_{0-24} (μg/mL/h)	79.4[d]	24.1	39.4	38.8	<0.001	36.7	26.1	<0.001
	F_r (%)	201.5	ND	100.0	ND	ND	93.1	ND	ND

AUC0-24: Area-under-the-curve between 0 and 24 h.
ND: Not determined.
[a] Pluronic® F127 polymeric micelles of concentration 10% w/v.
[b] Suspension produced by dispersing the content of EFV standard capsules in simple syrup.
[c] Simple solution of EFV in medium-chain triglyceride (MCT).
[d] Parameter of EFV-micellar system is significantly greater than that of the suspension and/or the MCT solution.

extensively used in Europe than in the U.S. that reveals divergences between both regulatory agencies [84]. Some groups explored the formation of complexes with different CDs to improve the aqueous solubility of ARVs [85,86]. However, the evaluation of *in vitro* data needs to be carefully pondered and not overestimated and complementary *in vivo* experiments need to be conducted because increased solubilization does not necessarily mean better bioavailability [86]. Also, the regulatory status of the different CDs has to be evaluated because they are considered safe for oral administration in children, but the experience is very limited [87].

At this point, three issues that might impact the clinical translation of innovative medicines deserve discussion. The first has to do with the lack of specific and unambiguous regulatory rules for evaluating technological, (bio)pharmaceutical or toxicological properties of nano-products, the second, with the capacity of a particular regulation framework to evaluate the appropriateness and safety of a clinical protocol, and the third with the readiness of a specific market to engage innovation. Innovation in therapeutics requires the constant training of personnel allocated to evaluate clinical protocols. Otherwise, the evaluation would not be robust enough and it might put the safety of the volunteers at risk. Conversely, if the "Precautionary principle" prevails owing to the lack of expertise of the regulation agency, patients might not benefit from products that are potentially effective and safe.

3.2. Tuberculosis

Tuberculosis (TB) is the second most deadly infection behind HIV, claiming 1.5–1.7 million annual deaths [88–90]. TB usually affects the airways but dissemination of the infection to other organs in untreated or resistant strains has been extensively reported [91]. The pharmacotherapy of standard TB combines rifampicin (RIF), isoniazid (INH), pyrazinamide and ethambutol over the first two months and RIF and INH over the last four months [92,93]. TB therapeutic success is intimately associated with high adherence to the administration regimens.

Table 3
EFV oral pharmacokinetics upon the administration of a single 200 mg dose to one adult healthy volunteer. The EFV concentration in the micelles was 20 mg/mL.

PK parameter	Capsule (200 mg)	Micelles (2%, 10 mL)
C_{max} (μg/mL)	1.26	3.14
t_{max} (h)	3	1
$t_{1/2}$ (h)	9.81	13.36
AUC_{0-24} (μg/mL/h)	19.19	28.59

Despite the fact that standard TB is a curable infection, it still represents more than 25% and 2.4% of the preventable and all deaths, respectively, in the world [94,95]. In developed countries, pediatric TB is only 5% of the total cases, while in developing ones the morbidity is 20–40%; the annual death toll of pediatric TB stands at 130,000 children [96]. As in HIV, children are a high-risk sub-population due to the difficult diagnosis and the lack of commercially available formulations with improved (bio)pharmaceutical performance [97], including RIF/INH FDCs. Indian companies have developed a number of double and triple FDCs, though none of them has reached clinical trials [98]. Moreover, some of these developments disregard that the conditions of the gastrointestinal tract could promote the degradation of some first-line drugs when they are administered by the oral route. For example, RIF is readily hydrolyzed to 3-formyl RIF SV in the acid gastric fluids, a product deprived of anti-TB activity *in vivo* due to limited oral bioavailability [99]. INH catalyzes this pathway, increasing the degradation rate in approximately 30–50% [100,101]. On the other hand, the co-administration of RIF and INH is recommended by the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Diseases to avoid mono-therapy, a practice that favors the development of bacterial resistance. Thus, the INH-mediated reduction of RIF oral bioavailability is a main global concern [102,103] in which new nanomedicines might play a relevant role.

The development of nanotechnology-based pediatric anti-TB products has been only addressed by a few research groups worldwide, mainly in developing countries [97]. The groups of Khuller and Swai investigated the encapsulation of several anti-TB drugs within polymeric and lipid nanoparticles and assessed their effectiveness in murine TB models by the oral route [104–108]. These studies were very robust because the preclinical evaluation was conducted in infected TB models. These nanomedicines are very promising to replace the current daily administration regimen by one single administration every 7–10 days. This would also facilitate the implementation of a Directly Observed Treatment, Short Course (DOTS), leading to greater adherence levels. Even though these developments were not primarily conceived for children, any successful bench-to-bedside translation would be beneficial for the pediatric population. At the same time, additional clinical trials in children are mandatory to comply with the PIP regulatory rule. Over the last five years, our group has investigated the encapsulation of RIF within different types of PMs [109,110]. Encapsulation increased the aqueous solubility of the drug up to 5.4 times, though RIF-loaded PMs underwent gradual secondary aggregation that resulted in poor physical stability over time. Thus, a cryo-protected lyophilization process was developed [111]. PMs not only chemically stabilized RIF under extreme acid conditions in the absence and presence of soluble

INH *in vitro* but also resulted in significantly greater oral RIF bioavailability (up to 3.3 times) with respect to a drug suspension blended with INH in a 3/2 weight ratio [112,113]. This nanotechnology platform could be employed to develop the first liquid pediatric FDC of RIF/INH.

Inhalation is commonly used in children to control asthma, bronchiolitis and bronchospasm. New developments in aerosol technology enable the fine control of the dose and the deposition, pointing out this route for the localized treatment of airway infections [114]. Most of the inhalation devices on the market have been developed for adult use, demanding some modifications to fit specific anatomo-physiological aspects of each pediatric sub-groups and to obtain the corresponding approval of the regulatory agencies. For example, pressurized metered dose inhalers could be used in children from birth by incorporating a spacer system and a face mask [22]. Conversely, dry powders would be acceptable in children older than 1 year of age [115]. Khuller and coworkers explored this route to target polymeric and lipid nanoparticles to alveolar macrophages, the intracellular mycobacterium reservoir [102,116–118]. Other groups followed a similar approach [119–123]. Aiming to capitalize on a previously developed platform, our group modified the surface of RIF-loaded PMs by coating them with chitosan and hydrolyzed galactomannan to improve the uptake by macrophages *in vitro* [124]. However, further *in vivo* assays would be required to assess the feasibility of this approach to treat the pulmonary form of the disease.

3.3. Malaria

Malaria is the parasitic infection with the greatest global morbidity and mortality [125]. Approximately 3.3 billion people were at risk of contracting malaria in 2011, populations living in sub-Saharan Africa accounting for 80% of the cases and 90% of the deaths [125]. Children under five years of age and pregnant women are the most severely affected subpopulations. Malaria is preventable and curable, though it caused the death of 660,000 children in 2010 [126] and it accounts for 20% of the pediatric mortality in Africa [125,126]. The first-line therapy of uncomplicated falciparum malaria in children is based on artemisinin combined therapies (ACTs) available in tablets that are difficult to swallow [127]. In this context, the WHO has called for the development of innovative oral ACT pediatric products [125]. The most relevant progresses include the development of suspensions, dispersible tablets and granules containing artemether/lumefantrine [128–130], artesunate/pyronaridine [131] and artesunate/mefloquine [132]. Different nanotechnologies have been investigated to develop innovative antimalarial products though only a few were specifically focused on the pediatric population [133]. CDs probably represent the most extensively used nanotechnology approach, especially aimed to mask the bitter taste of different antimalarial drugs. For example, Shah *et al.* improved the palatability of a liquid formulation of artemetherin [134]. Following a similar approach, complexes of primaquine phosphate were used to obtain powders for re-suspension [135].

4. Cancer

Cancer is the leading cause of death in children above 1 year of age in Europe and the USA [136]. Fortunately, more than 80% of children with cancer will survive in developed countries, though 40% will suffer long-term sequelae during adulthood. Most pediatric tumors are not found in adults because they originate from cellular populations that have not completed the process of terminal differentiation to specific organs or tissues [137]. Thus, although childhood is considered up to the age of 16/18 years [9,18], the biological definition of pediatric tumor (i.e., those tumors that arise from embryonic stem cells that reproduce the cells and environments from which they emerge, and occur during the infancy–childhood–puberty periods of the complex process of normal human development) may include adolescents and young adults [137]. Thus, most of the diseases included in this group are characterized by unique genetic alterations or biomarker expression. Examples of pediatric tumor-specific biomarkers are the EWS/FLI-1 fusion gene of Ewing sarcoma and the presence of the disialogangloside (GD2) in neuroblastoma [10,138]. In this framework, novel more effective diagnostic tools and more specific therapies are called for. However, the challenges for drug development are complex owing to the fact that (i) pediatric tumors are relatively very rare and (ii) the biology, the pathways and the cellular targets in pediatric cancers categorize them into different subtypes, reducing the number of potential volunteers that could be included in clinical trials and increasing the cost of the process [10,136].

Overcoming the mentioned difficulties, some nanomedicines have reached clinical trials in children with cancer (Table 4) [139]. The best example is provided by Abraxane® (Celgene), an albumin-stabilized nanoparticle formulation of paclitaxel (nab-paclitaxel) approved for adult refractory breast cancer and metastatic pancreatic cancer that was recently studied in preclinical pediatric solid tumor models including rhabdomyosarcoma, osteosarcoma and neuroblastoma [139]. As compared to the non-particulated drug, nab-paclitaxel showed superior activity against all tumors, associated with increased biodistribution of the drug in the tumor. This study has provided preclinical rationale for an ongoing Phase I clinical trial (NCT01962103) recruiting participants with pediatric solid refractory tumors. Before the Abraxane® study most of the pioneering pediatric nanomedicine research has been devoted to investigate the pharmacokinetics of nano-DDS with proven effectiveness in adults, in childhood tumors. One of the most popular and extensively investigated nanotechnologies to treat cancer is liposomes [140,141]. Table 4 summarizes different liposomal formulations of antitumorals under clinical evaluation in children [139]. Marina *et al.* assessed the tolerance and the pharmacokinetics of liposomal doxorubicin in 22 children with recurrent or refractory pediatric solid tumors such as neuroblastoma, rhabdomyosarcoma and pontine glioma [142]. The

Table 4
Antitumoral nano-formulations under clinical trial in children. *Reproduced and adapted from Ref. [141] with permission of Nature Publishing Group.*

FDA-approved liposomal formulation	Antitumoral	Indication/pediatric phase trial
Abraxane®[a]	Paclitaxel	Preclinical pediatric solid tumor models such as rhabdomyosarcoma, osteosarcoma and neuroblastoma
Doxil®	Doxorubicin	–Recurrent/refractory ovarian cancer, multiple myeloma in combination with bortezomib, –AIDS-related Kaposi sarcoma, and pediatric phase I/II in combination with temsirolimus in recurrent sarcoma
Depocyte®	Cytarabine	Lymphomatous meningitis and pediatric phase III of depocyte vs intrathecal triple therapy in acute lymphocytic leukemia
DaunoXome®	Daunorubicin	AIDS-related Kaposi's sarcoma and pediatric phase III in refractory/relapsed acute myeloid leukemia
FDA orphan drug designation of 1-annamycin	1-Annamycin	Recurrent/refractory adult ALL and pediatric phase I in refractory/relapsed acute lymphocytic leukemia or acute myeloid leukemia
Marqibo®	Vincristine sulfate	Recurrent/refractory adult lymphocytic leukemia in refractory sarcoma, neuroblastoma, Wilms tumor, leukemia, lymphoma, brain tumors (in children NCT01222780)
ThermoDox®	Doxorubicin	Unresectable hepatocellular carcinoma
CPX-351	Daunorubicin/cytarabine	Advanced adult lymphocytic and myeloid leukemias in refractory hematologic malignancies in children, adolescents and young adults

[a] From Ref. [139].

maximum tolerated dose over 4 weeks was 60 mg/m^2 every 4 weeks. A slightly higher dose of 70 mg/m^2 provoked mucositis. It is worth stressing that the authors included the patients (aged 4 to 21) in the study based on the pediatric nature of the disease, thus tumors that occur during human development are treated as a pediatric disease regardless of the age of the patient [137]. The rarity of pediatric cancer underscores the complexity of recruiting individuals for pediatric clinical trials and reaffirms the motivation to extend the use of nanomedicines to children to improve the efficacy of the treatment. Liposomal doxorubicin was also proposed to reduce the cardiotoxicity of the free drug in children, although data are still inconclusive [143]. Recently, the US-FDA approved the use of intratechal liposomal cytarabine for the prophylaxis or therapy of pediatric leukemia in the CNS [144]. A phase I study showed that the half-life of the drug in the cerebrospinal fluid of children was shorter than in adults, while the cytotoxic effects remained for at least 8 days as opposed to 3 h of the standard formulation. In addition, a maximum dose of 35 mg was well tolerated when combined with dexamethasone (0.15 mg/kg twice a day) over 5 days; the dose in adults is 50 mg. Remarkably, four children with refractory or relapsed CNS leukemia showed remission when treated with this nanomedicine [144]. The complementary treatment of opportunistic fungal infections in myelosuppressed children with liposomal amphotericin B has been also investigated [145].

Innovation entails both the encapsulation of approved drugs into nanocarriers to improve their (bio)pharmaceutical performance and the search of novel therapeutic agents. In this regard, nanotechnology applications aiming to deliver interference RNA (siRNA) to pediatric cancers are increasingly being explored to expand the therapeutic arsenal [141,146,147].

Owing the features of pediatric cancers that make unique this patient population, in the following sections we will describe the state-of-the-art at the interface of nanotechnology and the treatment of some of the most common and challenging pediatric cancers and discuss the perspectives of this field. We must mention that due to the relatively limited extent of the research conducted, most of the referenced studies are preclinical and they assessed the activity of nanomedicines in animal models of pediatric solid tumors. At the same time, a plethora of academic works investigated the interaction of innovative nano-DDS with cell lines of the same pediatric cancers *in vitro* as a proof of concept, but not with the specific aim of addressing them in children [148,149]. These studies were beyond the scope of the present review.

4.1. Neuroblastoma

Neuroblastoma is the most frequent extracranial pediatric solid tumor with special incidence in children below 2 years of age [150,151]. The course of the disease is heterogeneous and ranges from spontaneous remission to fast progression and death. Neuroblastoma is a cancer of simpatico-adrenal lineage, thus tumors can develop in any site of the sympathetic nervous system [151]. The standard treatment comprises chemo and immunotherapy, radiotherapy and surgery and it depends on the stage of the disease [151,152]. GD2 is ubiquitously present on the surface of neuroblastoma tumor cells and in normal tissues its presence is restricted to neurons and peripheral nerves [153]. Because the blood–brain barrier would protect neurons against GD2-targeted therapies, this molecule has been studied very extensively as a target for immunotherapy and drug delivery in this type of cancer. Neuroblastoma-targeted drug delivery has been attempted by a group at the Giannina Gaslini Children's Hospital (Genoa, Italy) in animal models (orthotopic tumors developed from the cell lines SH-SY5Y and HTLA-230) by means of anti-GD2 antibody-coated liposomes loaded with c-Myb antisense therapies [154,155], doxorubicin [156] and anti-ALK siRNA [157]. All the attempted targeted strategies achieved superior *in vivo* responses when compared to non-targeted ones. Other authors have tested the activity of the proapoptotic microRNA-34a loaded in anti-GD2 antibody-coated silica nanoparticles, showing similar results in the targeted therapy of neuroblastoma-bearing mice (NB1691 and SK-N-AS models) [158]. Interestingly, this study showed that the accumulation of fluorescent marker-loaded nanoparticles in tumor cells was 14-fold higher after the administration of anti-GD2 targeted nanoparticles as compared to non-targeted nanoparticles [155]. Multifunctionalized drug nanocarriers made of gold combined with paclitaxel and bearing an active targeting ligand (a humanized GD2 antibody, Hu14.18K322A) have been also designed to increase the antitumoral activity *in vitro* [159]. However, animal studies should be performed to gain further insight into the real potential of this formulation to treat the disease. Wang *et al.* reported on a similar strategy though based on carbon nanotubes modified with an anti-GD2 monoclonal antibody to target stNB-V1 cell line in vitro [160]. Even though the usefulness of this platform has been demonstrated *in vitro*, the hazardousness and toxicity of carbon nanotubes especially when administered by parenteral routes are a matter of discussion in different scientific forums. Since the non-toxicity of a nanocarrier is an essential property for its clinical implementation, their use in the treatment of children appears unrealistic [161]. At the same time, this work constitutes a valuable contribution to further understand the possible strategies to improve the treatment of highly-recurrent pediatric diseases. Despite the general interest on anti-GD2 targeting, nonspecific nanoparticle or macromolecule accumulation in neuroblastoma has also been explored. Pastorino *et al.* reported very good *in vivo* response of neuroblastoma-bearing animals to treatment with four-arm 40 kg/mol polyethylene glycol (PEG) linked via a glycine residue to SN38, a very potent but poorly water-soluble camptothecin (CPT) [162]. This new conjugate was soluble and cured a neuroblastoma model resistant to the standard of care chemotherapy (doxorubicin, cisplatin, vincristine and topotecan) and is currently under clinical trials (NCT01295697) [162]. In another approach, Qian *et al.* used NaYF4:Yb,Er/NaYF4/silica (core/shell/shell) up-conversion nanoparticles (70–80 nm) surface-decorated with gold nanoparticles (6 nm) for the photothermal ablation of SK-N-BE(2)-C neuroblastoma cells [163]. Nanoparticles were irradiated with near infrared and the up-converted green light was coupled to the gold plasmon for rapid heat conversion. This approach would enable a localized destruction of the tumor and a significant restriction of the adverse effects because the activity relies on the presence of the nanoparticles in the irradiated area. Lee *et al.* investigated the effect of Au(III) porphyrin-loaded unmodified and PEGylated lipid nanoparticles made of cetyl alcohol and Brij 78 in the NA2 neuroblastoma model [164,165]. Au(III) porphyrin is a poorly water-soluble compound shown to be 100-times more active against a wide range of cancers than cisplatin [166,167]. Encapsulation substantially reduced the systemic toxicity of the drug and prolonged the survival of tumor-bearing mice [164]. Remarkably, animals that received the free Au(III) porphyrin succumbed before the untreated individuals due to the intrinsic toxicity of the drug. To reduce accumulation in the liver and associated hepatotoxicity, nanoparticles were coated with PEG molecules of molecular weight 750 and 2000 g/mol [165]. The surface modification reduced the hepatic uptake to minimal levels, while it increased the accumulation in the tumor by the enhanced permeation and retention (EPR) effect. Schiffelers *et al.* engineered PEGylated polymeric nanoparticles of poly(ethylene imine) modified with Arg-Gly-Asp (RGD) at the edge of the PEG block for the delivery of siRNA sequences that inhibit VEGFR-2 expression [168]. Intravenous administration resulted in anti-angiogenesis and size reduction of N2A xenograft tumors (Fig. 1) [168]. Aryl hydrocarbon receptor siRNA has undergone clinical evaluation for the treatment of neuroblastoma owing to the fact that this receptor would play a tumorigenic role [147,169].

Nanomedicine is also being explored for cancer diagnosis. In this context, Lee and co-workers developed dual-mode nanoparticles comprised of a dye-doped silica core and multiple satellites of magnetic nanoparticles and surface-modified with HmenB1 antibody for the simultaneous high-performance magnetic resonance imaging (MRI) and fluorescence imaging of neuroblastoma expressing polysialic acids, the characteristic marker of this tumor that is present on the

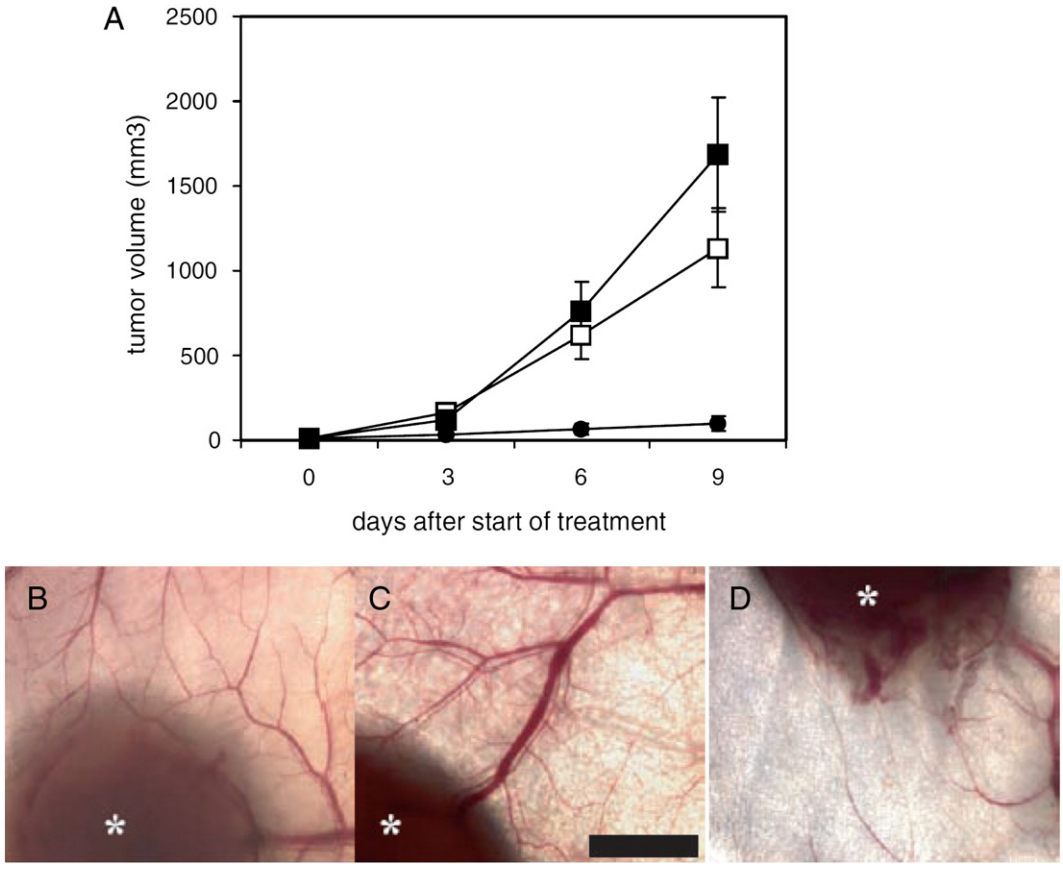

Fig. 1. Tumor growth inhibition by VEGFR2 siRNA nanoplexes. (A) Tumor growth inhibition by siRNA RGD-PEG-PEI (RPP)-nanoplexes. Mice were inoculated withN2Atumor cells and left untreated (open squares) or treated every 3 days by tail vein injection with RPP-nanoplexes with siLacZ oligonucleotide (filled squares) or siVEGF R2 (filled circles) at a dose of 40 mg per mouse. Treatment was started at the time-point that the tumors became palpable (~20 mm^3). Only VEGF R2-sequence-specific siRNA inhibited tumor growth, whereas treatment with LacZ siRNA did not affect tumor growth rate as compared with untreated controls (n = 5). (B–D) Neovascularization in tumors treated with siRNA RPP-nanoplexes. Representative tumors excised at the end of the tumor growth inhibition experiment (A) were examined using low magnification light microscopy. Trans-illumination of tumor and surrounding skin tissue shows strong neovascularization in mice left untreated (B) and mice treated with RPP-nanoplexes with siRNA-LacZ (C). In contrast, mice treated with RPP-nanoplexes with VEGFR2 siRNA showed low neovascularization and erratic branching of blood vessels (D). Asterisks indicate tumor tissue. Bar = 2 mm. *Reproduced and adapted from Ref. [168] with permission of Oxford University Press.*

neural cell adhesion molecules (NCAMs) (Fig. 2) [170]. Other groups proposed the technology of quantum dot-doped silica nanoparticles to follow up nanoparticles that also bear DNA-transfection capability [171].

4.2. Retinoblastoma

With a morbidity of approximately 1 in 18,000 births, retinoblastoma is the most recurrent malignant ophthalmic pediatric cancer and the third most common cancer in children, with greater incidence in developing countries where diagnosis is usually late [172]. A majority of cases (>80%) are in children below 3 years of age. The disease can be unilateral or bilateral, the former form being more common in older children (29–30 months) and the latter in younger ones (14–16 months) [173]. Retinoblastoma is originated in immature retinal cells that replace the retina and other intraocular tissues and displays a relatively high mitotic and apoptotic rate [173]. Regardless of the fact that retinoblastoma is a rare disease, it is a paradigmatic example of the consequences faced when there is a lack of basic elements of diagnosis and treatment as it is totally curable when diagnosed and treated at the early stages of development [174]. In this context, eye salvation and vision preservation are the primary goal in developed countries, while in developing countries retinoblastoma mortality is not uncommon. In advanced stages, enucleation is the standard therapy [175]. Several administration routes are being pursued to increase the bioavailability of antitumorals in the vitreous and to reduce their systemic exposure [176–178]. Even still to a limited extent, several attempts have been made to apply nanotechnology to the improvement of retinoblastoma therapy [179–181], most of them to fit active anti-retinoblastoma agents to the local delivery route and prolonged release regimens [182]. Kang and co-workers investigated the effect of subconjunctival carboplatin-loaded poly(amidoamine) dendrimer of generation 3.5 (PAMAM G3.5) in murine retinoblastoma [183]. Findings showed a statistically significant decrease of the tumor size in the treated eye with respect to the contralateral one.

Photodynamic therapy (PDT) is being studied as a potential future treatment for retinoblastoma [184]. In this case, the penetration and the selective accumulation of the photo-sensitizer in the tumoral cells are critical. Makky and co-workers synthesized glycodendrimeric porphyrins that would be recognized by the mannose (man) receptor, highly expressed in retinoblastoma cells [185–187]. Studies were conducted in a biomimetic model [188] and in a subcutaneous xenograft model of human retinoblastoma [187]. It remains unknown whether the described formulation would inhibit tumors in orthotopic location, likely isolated from systemic chemotherapy by the effect of the blood-retinal barrier. Following a similar rationale, Gary-Bobo *et al.* used silica mesoporous nanoparticles for one photon PDT (containing the porphyrin PS) loaded with the antitumoral CPT and modified with man or galactose (gal) for the active targeting of the nanocarriers to retinoblastoma Y79 cell line *in vitro* [189]. The combined approach led to greater cell death (Fig. 3) although the study does not contain *in vivo* data [189].

The overexpression of the antiapoptotic protein Bcl-2 in retinoblastoma has been shown to limit response to chemotherapy [190]. Another multidrug resistance (MDR) mechanism is associated with the activity of efflux pumps such as multidrug-resistance protein-1 (MRP-1) and lung resistance protein (LRP) that are fundamental in retinoblastoma. The development of nanotechnology strategies to overcome this specific resistance pathway is highly relevant. Following this concept, Das and Sahoo designed folate-modified dual nutlin-3a/curcumin-loaded poly(lactide-co-glycolide) (PLGA) nanoparticles for the therapy of retinoblastoma [191]. Nutlin-3a antagonizes murin double minute (MDM2), a negative regulator of p53, a tumor suppressor protein. Nutlin-3a is a substrate of MRP-1. On the other hand, curcumin modulates the expression and function of several efflux pumps, among them MRP-1. The conjugation of folate was aimed to target its specific epithelial cancer receptor. Folate-modified nanoparticles showed increased antitumoral activity with respect to the unmodified counterpart in a folate-expressing Y79 retinoblastoma cell line.

Other groups combined nanotechnology with external stimuli to improve the therapeutic outcome. For example, Kim and co-workers assessed the passage of gold nanoparticles of variable size through the blood-retinal barrier after i.v. administration [192]. The biodistribution in the retinal layers depended on the size. Thus, 100 nm nanoparticles did not cross the barrier, while 20 nm-size ones were detected in neurons (75%), endothelial cells (17%) and peri-endothelial glial cells (3%) [192]. In addition, these nanoparticles were not toxic. In this context, hollow nanoparticles could be used as drug carriers and in photothermal-ablation [193]. More recently, Wang *et al.* explored focused ultrasound to target the delivery of drugs to posterior pole retinoblastoma with thermo-sensitive drug nanocarriers that undergo heating upon to 39–44 °C irradiation, a stimulus that would trigger the release of an encapsulated drug [194].

4.3. Tumors of the central nervous system

4.3.1. Medulloblastoma

Medulloblastoma is the most common malignant pediatric neoplasm of the CNS. It is a very aggressive and invasive primary brain tumor localized in the cerebellum and the posterior fossa with a high tendency to metastasize [195]. The incidence is of approximately 9.6 cases per million children. The survival is currently in the 60–70% range, a significant improvement from the 30% reported in previous years. It is worth stressing that medulloblastomas in children and adults are genetically and histologically different [196] and they have been recently classified into four well defined molecular groups [197]. This challenges the selection of the right therapy and points out the need of pediatric clinical trials. The cellular and molecular mechanisms that initiate, maintain and sustain the progression of medulloblastoma remain unraveled though the Hedgehog signaling pathway has been related to the development of one type of the disease [198]. Some investigational drugs such as vismodegib that target this pathway are currently under clinical evaluation in adults and this could eventually expand the therapeutic repertoire in children [199]. Other cellular targets that have lately attracted the interest of oncologists for the treatment of medulloblastoma are microRNAs (miRNAs) [200]. These short non-coding RNA sequences (22 nucleotides) regulate transcriptional and post-transcriptional stages of gene expression [201]. Regardless of the therapeutic potential of these molecular targets, the first investigations at the interface of medulloblastoma and nanotechnology employed commercially available liposomal formulations approved for the treatment of adult tumors. Also, because some subtypes of medulloblastoma occur in adults, such patients were the first suitable to enter clinical trials, from both a regulatory and an ethical perspective. For example, Boiardi *et al.* evaluated the safety and effectiveness of daunorubicin-loaded liposomes in adult patients with advanced

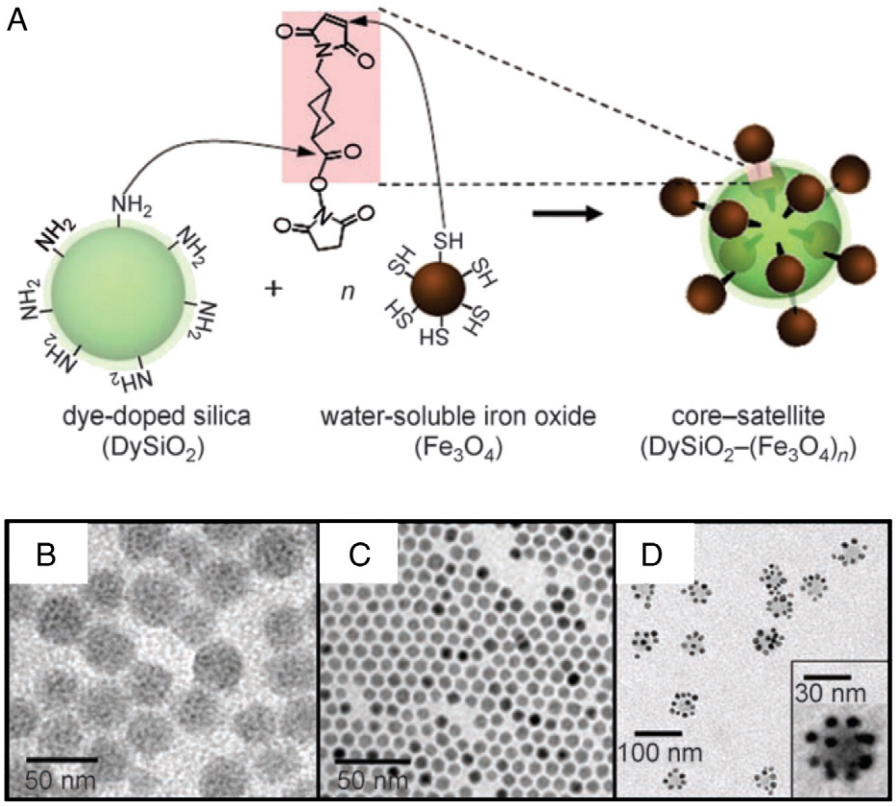

Fig. 2. (A) Schematic diagram for the synthesis of core–satellite $DySiO_2$–$(Fe_3O_4)_n$ nanoparticles. (B–D) TEM images of rhodamine-doped silica ($DySiO_2$), iron oxide (Fe_3O_4), and core–satellite $DySiO_2$–$(Fe_3O_4)_n$ nanoparticles.
Reproduced and adapted from Ref. [170] with permission of Wiley & Sons.

recurrent gliomas and medulloblastoma [202]. Results in medulloblastoma were partially positive. In the early 2000s, Piccaluga et al. reported on the activity of a similar formulation (DaunoXome®) for the clinical evaluation in relapsed meningeal acute myeloid leukemia and suggested that since liposomes surpass the blood–brain barrier they would be useful also for the treatment of other tumors of the CNS [203]. Since then, only countable preliminary studies explored nanotechnology platforms to improve the therapy of medulloblastoma [204,205]. In a recent work, Zucker and Barenholz developed a liposomal combination of vincristine and topotecan co-encapsulated in the same nanocarrier to co-deliver them to Daoy human medulloblastoma cell line with positive results [205]. Studies in a relevant *in vivo* model are pending. The lack of an effective therapy has also motivated researchers to study alternative drugs. Lim and co-workers developed curcumin-loaded nanoparticles made of *N*-isopropylacrylamide, vinylpyrrolidone and acrylic acid (NanoCurc®) and showed a dose-dependent decrease in the growth of Daoy and D283Med cell lines *in vitro* [206]. An interesting contribution of this work was the characterization of the changes in CD133-positive stem-like tumor subpopulations in spheres, an *in vitro* model that is more representative of the clinical disease [206]. Moreover, nanocurcumin down-regulated the expression of IGF-1, STAT3 and Gli1 in Daoy cells, three proteins that are associated with the initiation and the growth of medulloblastoma [206]. The relevance of *in vitro* assays should not be overestimated, especially because this type of cancer is characterized by the fast dissemination and the invasiveness of its cells in different areas of the CNS and the conservation of an intact blood–brain barrier. On the other hand, the difficult reproduction of the disease in animal models demands intermediate stages to evaluate potentially useful therapeutics. In this framework, Meng and co-workers developed an *in vitro* 3D model of brain tumors [207,208]. The next challenge would be the development of an appropriate xenograft model.

During the last years, the field of theranostics (therapy + diagnostics) emerged as a new concept to address disease in one single treatment [209–211]. In this context, Sun *et al.* used Fe_2O_3 superparamagnetic nanoparticles surface-modified with PEG and chlorotoxin, a peptide isolated from scorpion venom that shows great affinity for neuroectodermal cells, to co-deliver an antitumoral drug (methotrexate) and an MRI agent to medulloblastoma (D283) and glioma (9L/lacZ) cell lines and to a murine xenograft glioma model [212]. The same ligand was used to target a nanovector/DNA complex to Daoy cells [213].

4.3.2. Astrocytoma

Astrocytoma is another kind of intracranial brain tumor originated in gliomal cells called astrocytes. It can develop in any zone of the brain and also in the spine. Based on the recognition of anaplasia (nuclear atypia, cell pleomorphism, mitotic activity, endothelial hyperplasia and necrosis) and histological analysis, the WHO classifies astrocytomas according to growing malignancy into grades I (pylocytic and subependymal giant cell astrocytomas), II (pylomyxoid, peomorphic xanthoastrocytomas and diffuse astrocytomas), III (anaplastic astrocytoma) and IV (glioblastoma multiforme) [214–216]. Low-grade astrocytomas (grades I and II) are benign tumors and are among the most common pediatric cancers [217]. The prognosis is good, though it depends on the brain mass lost after surgical resection. Owing to the heterogeneity of these tumors, the development of innovative systems is tricky, as they could be effective in one type and ineffective in the other. This also challenges the conduction of clinical trials. Only a few preliminary studies addressed the use of nanotechnology to treat this type of cancer, most of them focusing on high grade astrocytomas that are more characteristic of the adult population. The most common nanocarrier was PEGylated and ligand-conjugated liposomes [218] usually loaded with campthotecin [219], lomustine [220] or doxorubicin [221–223].

4.4. Musculoskeletal tumors

This group of malignant tumors includes Ewing sarcoma (EWS), osteosarcoma and rhabdomyosarcoma, among others, and comprises approximately 10% of the new cases of cancer in children, adolescents and young adults [224].

4.4.1. Ewing sarcoma

EWS is a malignant cancer of children and adolescents that has a genetic basis and is characterized by small, round, blue cells that can be found in bone and more rarely in soft tissue [225]. EWS presents a tumor-specific EWS/FLI-1 fusion oncogene, a product of the translocation of chromosomes 22 and 11 [226–228]. The current treatment combines chemotherapy, irradiation and surgery. The incorporation of nanotechnology tools opens new treatment opportunities [229]. Because small molecule drug development against EWS/FLI1 has remained elusive, alternative approaches have explored the potential of nanotechnology to target EWS/FLI-1 with siRNA. Toub *et al.* encapsulated a siRNA

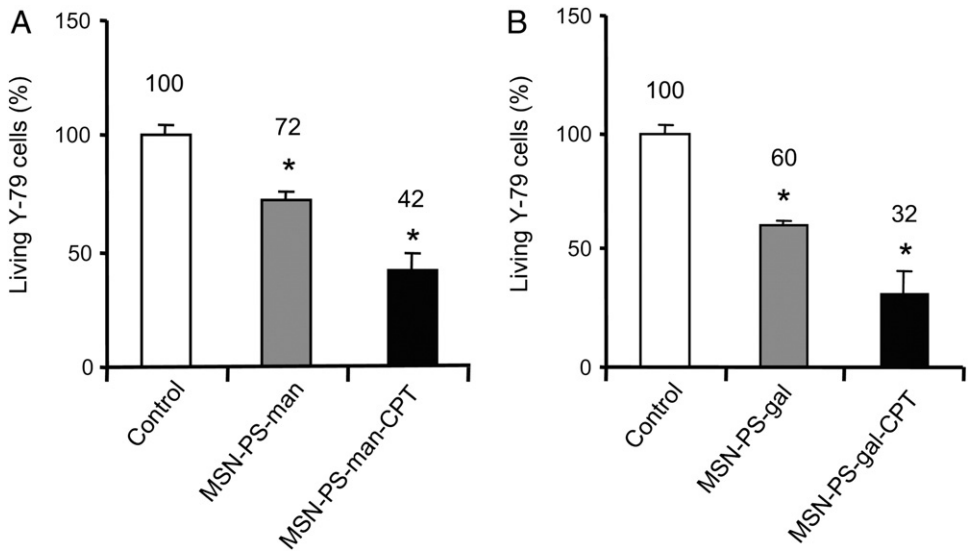

Fig. 3. Effect of combined PDT and drug delivery on retinoblastoma Y79 cells incubated or not (control) with 20 μg/mL of (A) MSN-PS-man or MSN-PS-man-CPT; or (B) MSN-PS-gal or MSN-PS-gal-CPT for 24 h and then submitted to laser irradiation ($\lambda = 650$ nm, 90 J/cm^2). Cells were allowed to grow for 2 days and cell viability was quantified. Data are mean ± S.D. of 3 independent experiments. *$p < 0.05$ indicates statistically different from control.
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within non-ionic poly(isobutylcyanoacrylate) nanocapsules with an aqueous core to block the expression of this fusion gene [227]. This formulation inhibited the growth of EWS in nude mice in a dose-dependent manner owing to the under-expression of EWS/FLI-1 (Fig. 4) [227]. Thus, the less frequent administration of a smaller dose for a shorter period led to a greater tumor growth inhibition than the free drug. Alhaddad and collaborators followed a similar approach, though they used nanodiamonds pre-coated with poly(ethylene imine) or poly(allylamine hydrochloride) that enabled the adsorption of siRNA onto the surface [228]. Their capacity to inhibit the expression of EWS/FLI-1 fusion gene in two cell lines, NIH/3T3 EF and A673, was assessed with positive results. Loss of expression of the fusion gene was associated with the complete arrest of the growth and the increase of apoptosis of Ewing tumor cells [230].

4.4.2. Rhabdomyosarcoma

Rhabdomyosarcoma is a type of connective tissue cancer that stems from skeletal muscle progenitor cells and it is characteristic of childhood [224,231,232]. It displays different histological subtypes (embryonic and alveolar being the most common in children) and it can be found in any body site. Approximately 16% of the cases show metastasis in the lung, lymph nodes, bone marrow and bone [232]. Depending on the form and the stage of the disease, the treatment could comprise chemo and radiotherapy and surgery.

The development of nano-DDS for the therapy of this type of cancer is in its infancy and only isolated studies have been reported. In a pioneering work, van Bree et al. investigated the antitumoral activity of thermostable DaunoXome® liposomes combined with hyperthermia (1 h, 43 °C) in rats bearing R-1 rhabdomyosarcomas [233]. The accumulation in the tumor was similar for the free and the encapsulated drug. Conversely, thermal treatment increased the accumulation by 5.4-fold probably due to increased drug extravasation by the EPR effect. These early results were resumed in a more recent work by Morita and co-workers where doxorubicin-loaded thermo-sensitive liposomes were heated with infrared-A radiation [234]. In this case, tumors were heated prior to the i.v. infusion of the formulations and heated 1 h post-administration at 42.5 °C. This combination delayed and inhibited the growth of rhabdomyosarcomas in rats and reduced the systemic toxicity.

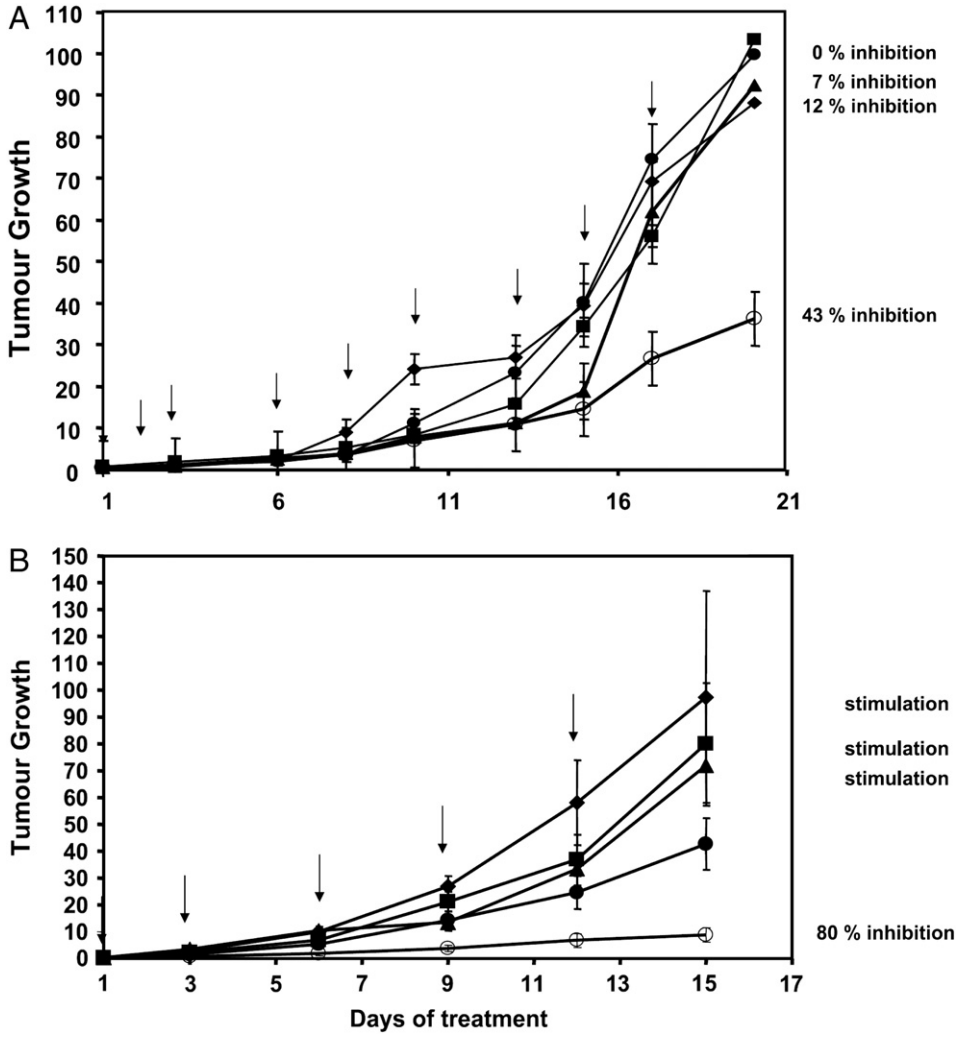

Fig. 4. Inhibition of growth of EWS/FLI-1-expressing tumor in nude mice by siRNA-loaded poly(isobutylcyanoacrylate) nanocapsules (NC). (A) Cumulative dose of 0.8 mg/kg (1.44 nM) administered by intratumor administration for 20 days. Arrows correspond to the days of treatment (1, 2, 3, 6, 8, 10, 13, 15, and 17). (B) Cumulative dose of 1.11 mg/kg (2 nM) administered by intratumor administration for 15 days. Arrows correspond to the days of treatment (1, 3, 6, 9 and 12). The effect of antisense siRNA in nanocapsules on tumor growth in nude mice is expressed relatively to tumor volume on day 1 according to the following formula: tumor volume at day x/tumor volume at day 1. All siRNA, free or encapsulated (NC), were used at a dose of 88 mg for each injection. The cumulative dose of siRNA was 0.8 mg/kg (1.44 nmol). ○ NC siRNA-AS; ▲ NC siRNA-Ct; ■ siRNA-AS; ◆ siRNA-Ct and ● saline. Bars indicate the standard deviation of the mean for six mice.
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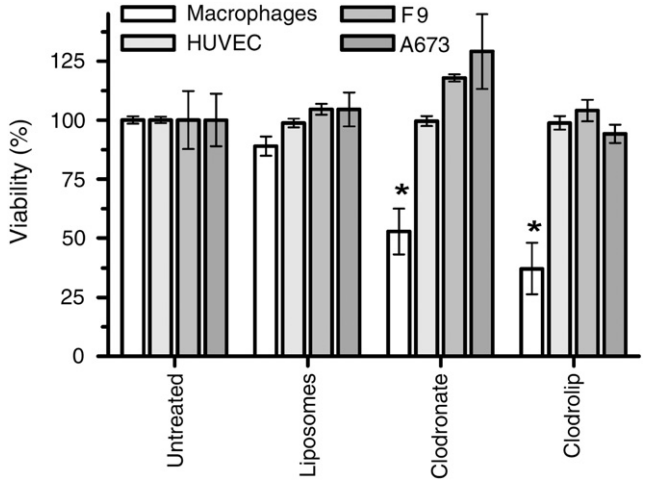

Fig. 5. Cytotoxicity of clodronate or clodronate liposomes (clodrolip) on different cells *in vitro*. Macrophages, HUVEC, F9 and A673 cells were cultured in the presence of 1 mg/mL clodronate or clodronate liposomes (clodrolip) for 6 h. Results are means ± s.e.m. (n = 3). *$P < 0.05$ versus untreated cells.
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The use of external stimuli to improve the performance of nanomedicines has been previously described for metallic nanoparticles [192,193], though other research groups profusely explored the combination of antitumoral-loaded nanocarriers (e.g., PMs) with ultrasound localized heating to trigger the release of the encapsulated drug and the increase of cell membrane permeability [235–241]. Zeisberger *et al.* followed a more original approach and they depleted tumor-associated macrophages and blocked tumor angiogenesis with clodronate-loaded liposomes that were combined with VEGF antibody in a human A673 rhabdomyosarcoma mouse tumor model [242]. *In vitro* preliminary studies showed that the inhibitory activity was specific on macrophages and no effect was observed on HUVEC, murine teratocarcinoma F9 and human rhabdomyosarcoma A673 cell lines (Fig. 5) [242]. Antitumoral assays showed the benefit of the clodronate liposomes and anti-VEGF combination (Fig. 6) [242].

4.4.3. Osteosarcoma

Osteosarcoma is the most common bone sarcoma in children and adolescents and it comprises a series of high-grade histologic variants [224,231]. Surgery and complementary chemotherapy are recommended since the early 1980s. Prognosis is intimately related to the localization of the tumor and relapse rates are relatively high in patients with localized (30%) and metastatic (80%) disease [231]; relapse is usually in the lung. This type of cancer probably concentrates the most intense nanotechnology research activity of all the pediatric cancers addressed in this article [243]. Liposomes loaded with a variety of antitumoral (e.g., 9-nitrocamptothecin, cisplatin, curcumin) and immune-stimulatory drugs (e.g., muramyl tripeptide (MTP) or mifamurtide) attracted a great deal of attention probably because there are commercially available products employing this technological platform such as Doxil® with a long tradition in the treatment of cancer [244–250]. In general, the studied administration route was parenteral, though in specific cases the treatment of lung metastases by inhalation was also assessed [246,247]. For example, MTP phosphatidylethanolamine, a synthetic lipophilic analog of muramyl dipeptide, in liposomal formulation (Mepact®, Takeda) is a novel nanomedicine that is up-taken by monocytes and macrophages which undergo activation and become tumoricidal [248]. Thus, it is used in combination with regular chemotherapy based on cisplatin, methotrexate, doxorubicin and/or ifosfamide. In a recent study, Dhule *et al.* evaluated the antitumoral activity of a curcumin/2-hydroxypropyl-γ-cyclodextrin complex encapsulated within liposomes against KHOS osteosarcoma cell line *in vitro* and in a xenograft osteosarcoma model *in vivo* [250]. In another nanotechnology twist, Drug-in-CD-in-Liposome (DCL) delivery

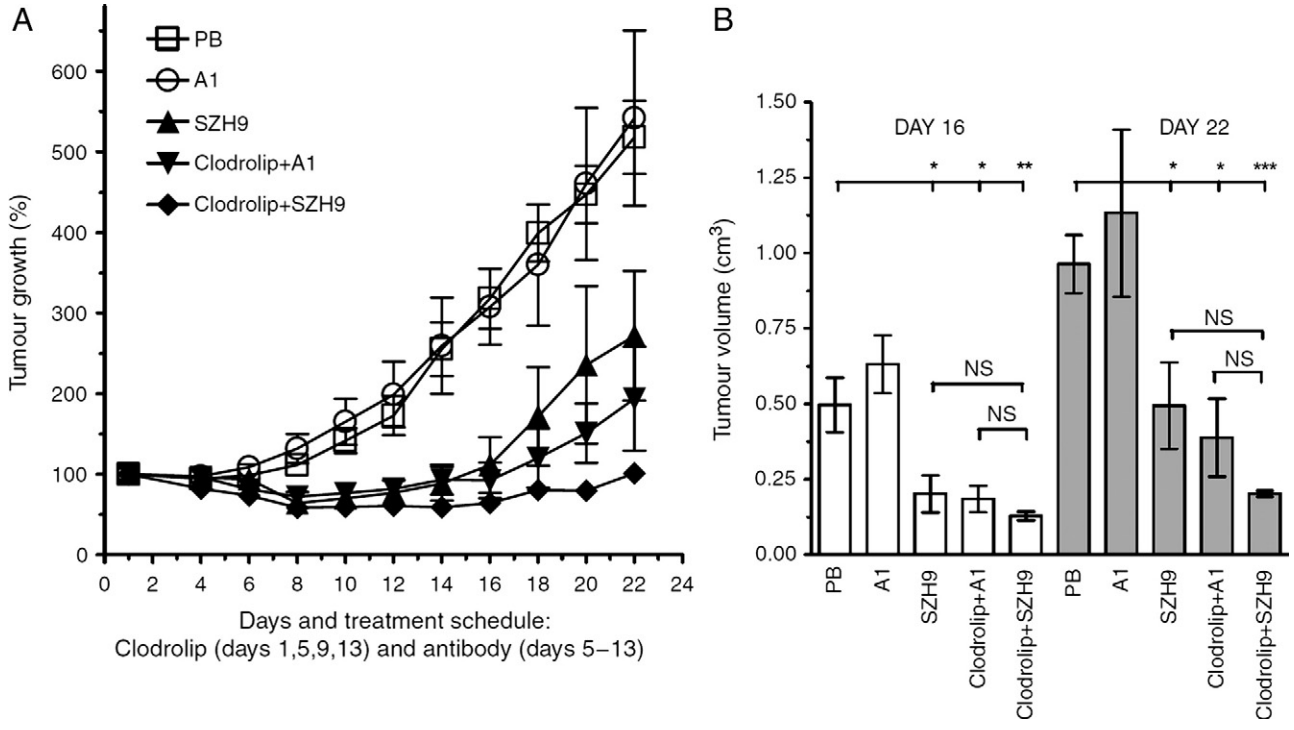

Fig. 6. Combination of clodrolip with anti-VEGF (SZH9) enhances tumor-associated macrophage depletion and shows improved tumor inhibition in a xenogenic human A673 rhabdomyosarcoma model. (A) Tumor growth inhibition by the combination treatment in A673 rhabdomyosarcomas. Tumor-bearing mice (n = 6–8) were treated with phosphate buffer (PB), unspecific control antibody (A1), specific antibody (SZH9) (days 5–13, i.v.), clodrolip + A1 or clodrolip + SZH9 (days 1, 5, 9 and 13, i.p.). (B) Tumor growth inhibition measured at days 16 (open bars) and 22 (gray bars) showing the tumor volumes as calculated averages ± s.e.m.
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systems have been proposed to improve the encapsulation efficiency of liposomes [251]. Curcumin induced apoptosis with an increase of the concentration of markers such as cleaved caspase and poly(ADP-ribose) polymerase and autophagy in vitro. Results in vivo followed a similar trend (Fig. 7) [250]. Again here, the use of approved pharmaceutical excipients was aimed to pave the way to a potential clinical study and represents an added value of this development.

The search for novel therapeutic agents to improve the outcomes motivated Tran *et al.* to evaluate the effect of selenium nanoclusters grown on titanium surfaces on the proliferation of normal and tumoral osteoblasts [252,253]. Findings indicated that selenium nanoclusters promoted the proliferation of normal osteoblasts, while the opposite was true for tumoral osteoblasts in both mono- and co-culture assays. In addition, the higher the selenium density on the surface, the stronger the antitumoral effect observed [253]. The activity was related to the release of soluble selenium that is cytotoxic [252]. In this context, this type of bone implants could be inserted intratumorally upon the resection of the tumor to eliminate residual tumoral cells. Similarly, doxorubicin-loaded chitosan/dipotassium orthophosphate hydrogels and chitosan/dextran sulfate microparticles were designed to sustain the local release of doxorubicin, the anti-osteosarcoma gold-standard, to primary osteosarcoma and secondary metastasis in a sustained regimen [254,255]. The hydrogel resulted in a significant reduction of both primary and secondary osteosarcoma in a clinically relevant orthotopic model (Fig. 8) and reduced lung macrometastasis (Fig. 9) and cardiotoxicity [254]. Following a similar trend, the treatment of mice with doxorubicin-loaded microparticles also reduced the development of osteosarcoma in a murine model (Fig. 10) [255]. Furthermore, the treatment with the microparticles resulted in weight maintenance and less side-effects such as heart failure or dry skin as compared to doxorubicin solution [255].

The use of combinational therapy and external stimuli was mentioned above. To control the growth of Os515 osteosarcoma implanted in Syrian male hamsters adriamycin-loaded magnetic liposomes were accumulated in the tumor site by applying an external magnetic field [256,257]. When combined with a magnetic force, the antitumoral activity of adriamycin increased significantly with respect to the free

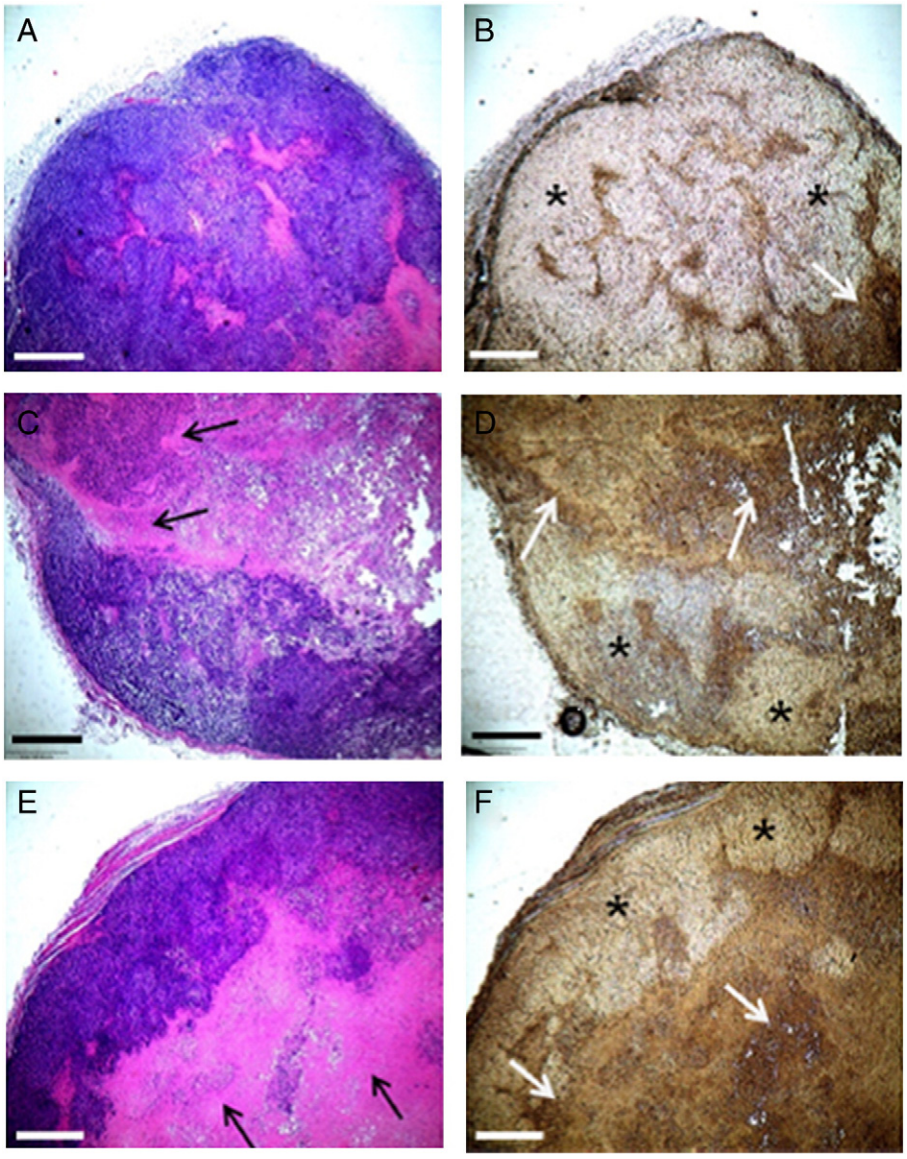

Fig. 7. Curcumin-loaded liposomes induce apoptotic cell death in osteosarcoma xenograft model. Tumors treated with (A,B) empty liposomes, with (C,D) conventional curcumin liposomes and with (E,F) hydroxypropyl-γ-CD-curcumin liposomes. Subfigures A, C, E represent hematoxylin and eosin staining for histopathology of tumor, whereas B, D, F are the images of TUNEL staining for detection of apoptosis. (* indicates high proliferative area and white arrows indicate apoptotic area). All the scale bars indicate 500 μm.
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drug solution and the liposomes without magnetic stimulus. The combination of liposomal doxorubicin with hyperthermia was also assessed [258]. It is interesting to note that the use of drug-free magnetite cationic liposomes induced hyperthermia and the pronounced remission of experimental osteosarcoma in hamsters when exposed to an alternating external magnetic field [259]. Ueno *et al.* coupled ultrasound to bubble liposome (a new kind of liposome that entraps an ultrasound imaging gas, perfluoropropane, and that is more sensitive to low-power ultrasound) to increase the efficacy of free doxorubicin in a murine osteosarcoma xenograft [260]. The combination prolonged the survival with respect to non-treatment and free doxorubicin. Moreover, the required dose in the combination was 1/5 of that of the free drug to achieve a similar result and the intratumoral drug concentration was 1.5 times greater than the control. Davies *et al.* combined liposomal doxorubicin with radiotherapy and demonstrated the increased uptake and biodistribution of liposomes with respect to sole chemotherapy in osteosarcoma xenografts in athymic mice [261].

Heterogeneous distribution of antitumorals in solid tumors represents a drawback of the therapy. Alteration of the extracellular matrix by the intratumoral administration of hyaluronidase induced a transcapillary pressure decrease probably due to the degradation of the extracellular matrix that resulted in a 4-fold increase of the tumor uptake of liposomal doxorubicin [262]. Conversely, the microvascular pressure remained unchanged. This improvement was greater than the one attained with radiation [261]. Sun and co-workers synthesized dextran-*g*-poly(ethylene imine) copolymers for the co-delivery of adriamycin and plasmid DNA into MG-63 and Saos-2 osteosarcoma

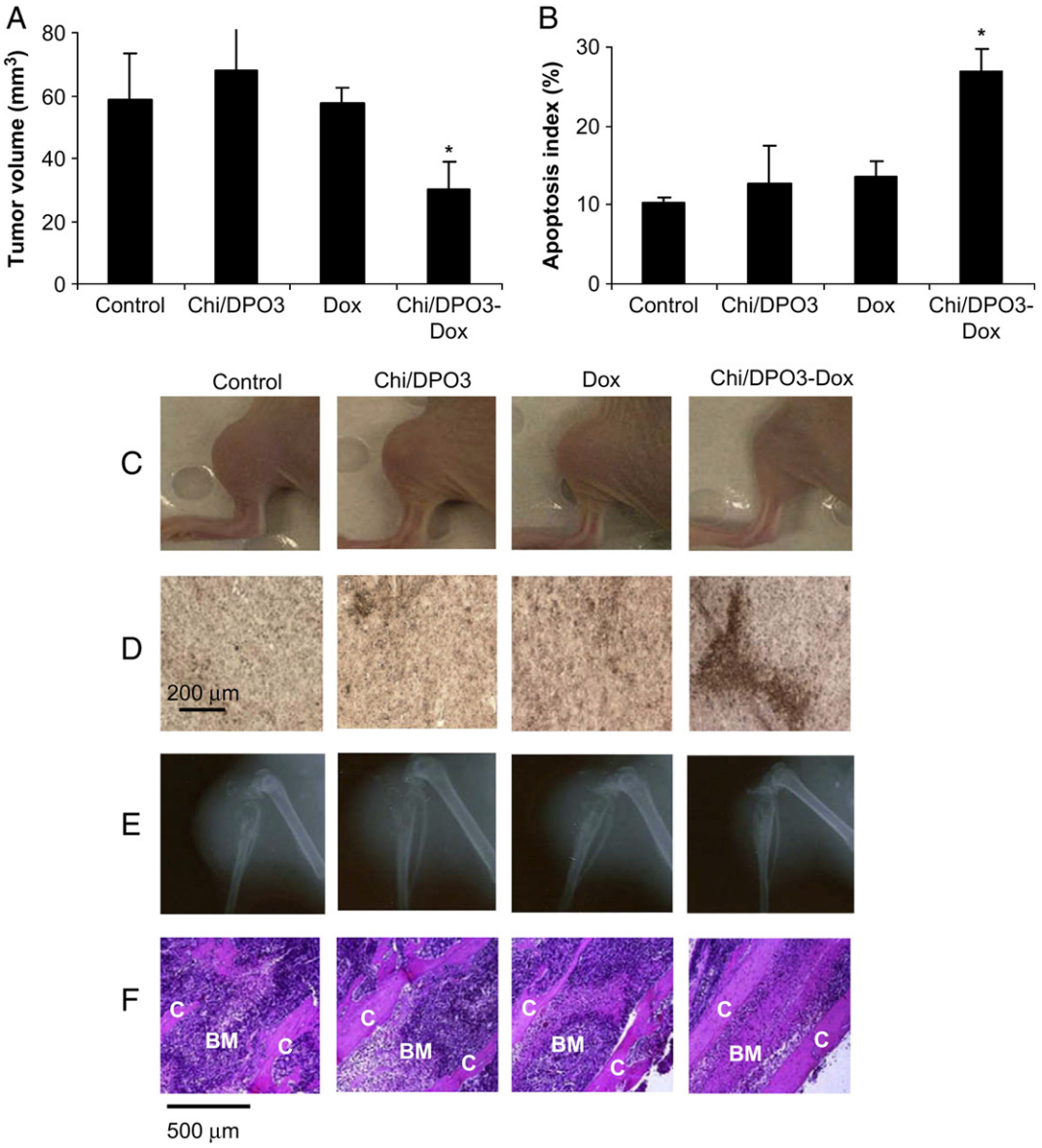

Fig. 8. Inhibitory effect of a doxorubicin-loaded hydrogel (Chi/DPO3–Dox) on in vivo tumor growth in mice after 12 days post-treatment. (A) Tumor growth inhibition graph, $^{*}P < 0.05$ vs. control group. (B) Tumor apoptosis graph, $^{*}P < 0.05$ vs. control group. (C) Primary tumors, showing significantly large tumors in samples from control, Chi/DPO3 and Dox groups, but nearly nil tumors in sample from Chi/DPO3–Dox group. (D) TUNEL-stained apoptotic tumor tissues, showing much less apoptotic cells (dark brown) in tumor tissues from control, Chi/DPO3 and Dox groups, compared to tumor tissue from Chi/DPO3–Dox group (magnification ×40). (E) X-ray images, indicating severe bone lysis in samples from control, Chi/DPO3 and Dox groups and only mild osteolysis observed in sample from Chi/DPO3–Dox group. (F) Histological analysis of tumor growth, showing that tumor cells severely degraded bone cortex and invaded bone marrow in samples from control, Chi/DPO3 and Dox groups, with less bone degradation in sample from Chi/DPO3–Dox group (BM: bone marrow, C: cortex) (magnification ×200). Reproduced from Ref. [254] with permission of Elsevier.

cells [263]. Even if *in vitro*, this study was a proof of concept because it assessed the transfection of the green fluorescent protein gene. On the other hand, this technology could be combined with c-jun gene knockdown therapy that increased the apoptosis of osteosarcomas [264] or the transfection of a plasmid expressing pigment epithelium-derived factor, a potent anti-angiogenic factor, that decreased primary tumor growth and reduced bone lysis and the establishment of lung metastasis [265]. Another anti-angiogenic coadjuvant therapy to prevent osteosarcoma metastasis was reported by Dutour *et al.* [266]. In this work, an endostatin-coding plasmid was encapsulated within cationic liposomes and tested in an orthotopic osteosarcoma model in rat. This treatment delayed the tumor growth and prevented the development of lung metastases.

Active targeting is another attractive approach to improve the therapeutic index in osteosarcoma. Anada *et al.* designed doxorubicin-loaded biphosphonate-conjugated liposomes with great affinity for hydroxyapatite and consequently for bone tissue and showed increased cytotoxic activity against human osteosarcoma MG63 cell line [267]. However, from this study, the ability of this nano-DDS to accumulate in tumors to a greater extent than in normal bone tissue is unclear. More recently, Wu and Wan carried out a more comprehensive study with a similar system in UMR106 and SOSP-M cell lines *in vitro* and in a UMR106 osteosarcoma model in Sprague–Dawley rats and a SOSP-M pulmonary metastatic osteosarcoma model in nude mice [268]. Greater anti-osteosarcoma activity and lesser toxicity were observed. The expression of human epidermal growth factor receptor-2 (HER2) has been associated with metastasis and survival of osteosarcoma patients [269]. Thus, this receptor could be exploited to target drugs to metastatic tumors [270]. A chimeric molecule of HER-2 single-chain specific antibody and domain II of *Pseudomonas exotoxin A* was used to block osteosarcoma growth and metastasis in SOSP-9607-E10 xenografts in Balb/C athymic mice [271]. Transferrin-2 is another receptor highly expressed in cancerous cells [272]. Nakase *et al.* evaluated a p53 gene therapy in osteosarcoma HOSM-1 cell line using cationic transferrin-conjugated liposomes [273]. Results showed increased apoptosis and cell growth inhibition both *in vitro* and *in vivo* when the nano-DDS was locally administered. In a very elegant work, Segal *et al.* synthesized a multifunctional conjugate of *N*-(2-hydropropyl) methacrylamide copolymer (HPMA), alendronate (ALN) and TNP-470, a potent inhibitor of methionine animopeptidase-2 that results in endothelial cell cycle arrest and inhibition of tumor angiogenesis for improved anti-osteosarcoma efficacy [274]. The conjugate resulted in significantly greater activity than free ALN/TNP-470 (Fig. 11) [274].

5. Perspectives

According to the market forecasting company BCC Research, the global nanomedicine market has been growing steadily, reaching a value of $72.8 billion in 2011, with anticancer agents as the market leader [275]. The market is expected to increase at an annual growth rate of 12.5% until 2016, reaching a value of $130.9 billion. In this global context, nanomedicine emerges as a promising tool to also optimize the therapy of pediatric diseases [276]. An expression of this potential and vision was the development of the pioneering NanoPediatrics Program

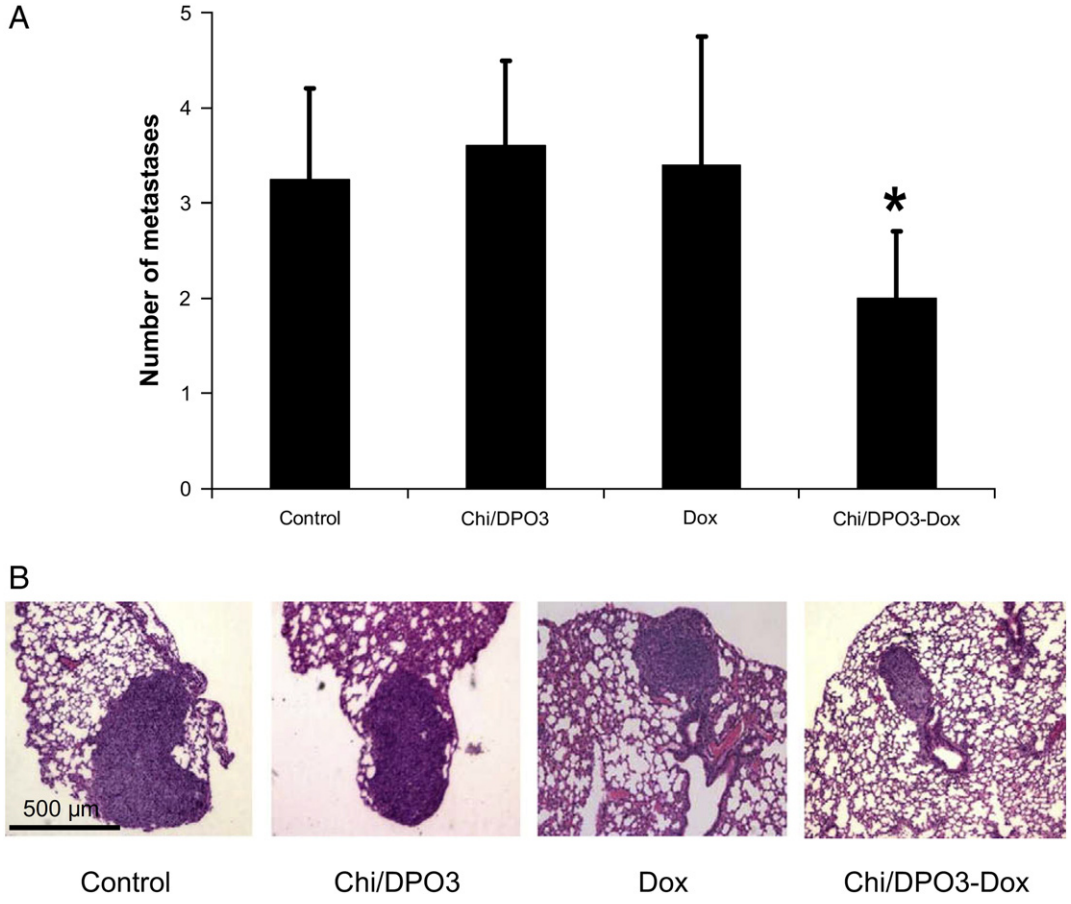

Fig. 9. Inhibition of lung macrometastasis in mice after 12 days post-treatment. (A) Lung macrometastasis inhibition graph, $^*P < 0.05$ vs. control group. The number of macrometastases was enumerated on the surface of all lobes of each lung against a white background. (B) Histological analysis of lung metastasis (P: parenchyma, T: tumor) (magnification ×40). Reproduced from Ref. [254] with permission of Elsevier.

at the Mattel Children's Hospital of the University of California, Los Angeles in 2008 [277] and the foundation of the Center for Pediatric Nanomedicine, a joint venture of the Emory University School of Medicine and the Children's Healthcare of Atlanta, in 2011 [278]. Other specialized institutions such as The Australian Centre for Nanomedicine at the University of New South Wales (Sydney, Australia) devoted one of its pioneering projects to the development of new treatments for neuroblastoma, the most common cancer of children under five years of age [279]. All these are signals of the relevance of this research avenue. At the same time, we must be realistic and stress that the pace of bench-to-beside translation is a long and intricate process and that the number of nanopharmaceutical products on the market is relatively small compared to the research resources allocated in this field. Advances in pediatric nanomedicines might be improved by the focused

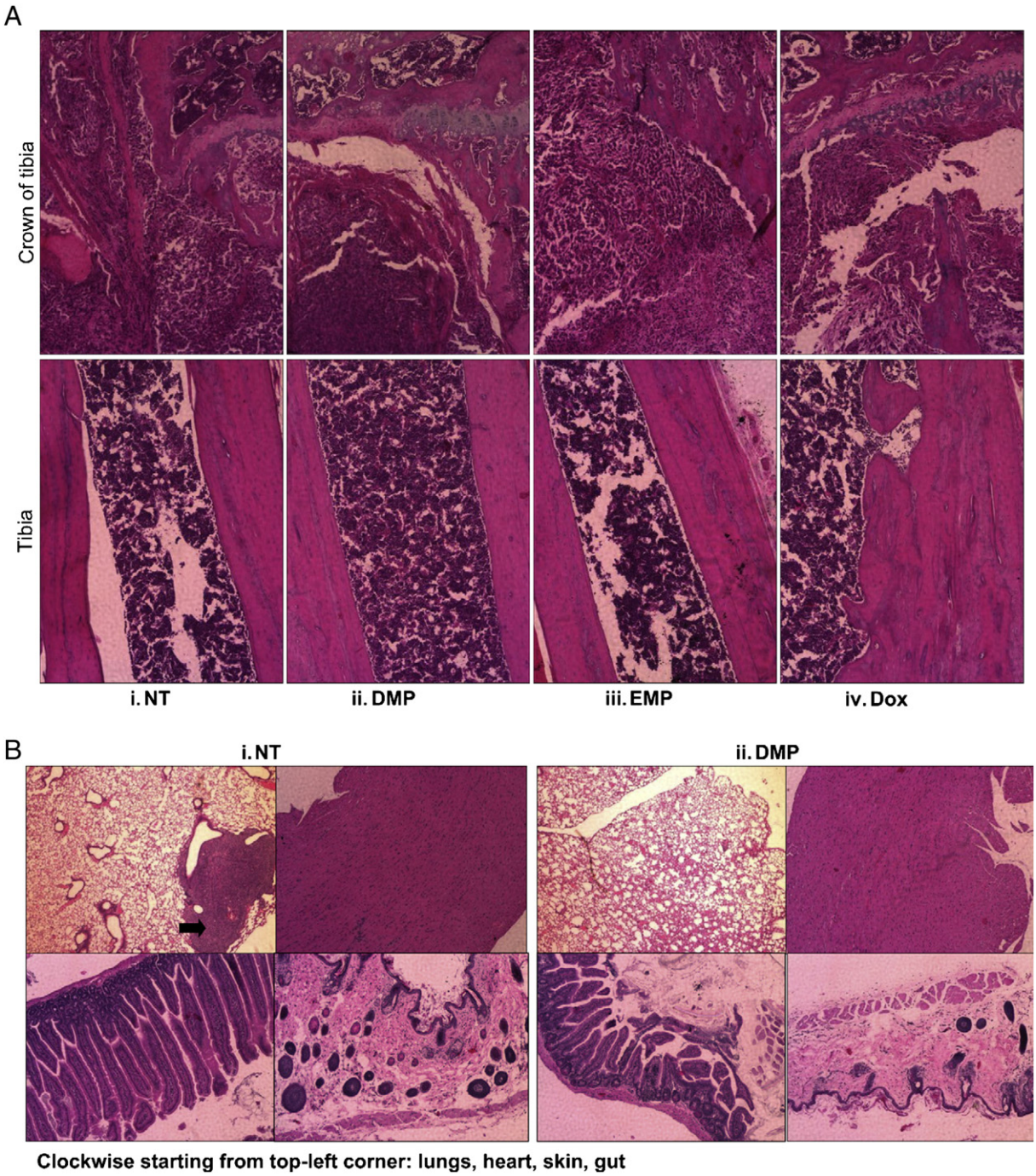

Fig. 10. (A) H&E sections of mice tibia (100× magnification) from representative treatment groups. Widespread invasion of tumor cells in the crown of the tibia due to broken growth plate cartilage (GPC) and epiphyseal cartilage (EC) in nil-, empty microparticles (EMP)-, and DOX-treated mice. Doxorubicin microparticles (DMP)-treated mice appeared to have intact GPC and EC, preventing further invasion of tumor cells. Note bone marrow depletion in nil-, EMP-, and DOX-treated mice, while DMP-treated mice were unaffected. Note also the bone abnormalities in the DOX-treated mice. (B) H&E sections of accessory organs implicated during tumor growth and treatment. Clockwise starting from the top left-hand corner: mice lungs, heart, skin and gut from representative treatment groups. Note the presence of tumor metastases in the lungs as determined by dark purple staining (black arrow). Small foci in the heart myocytes with cytoplasmic vacuolization (white arrow) and between myocyte layers. Loss of organized skin structure (white arrowhead). Disintegration of gut lining as seen as serrated morphology (black arrowhead).
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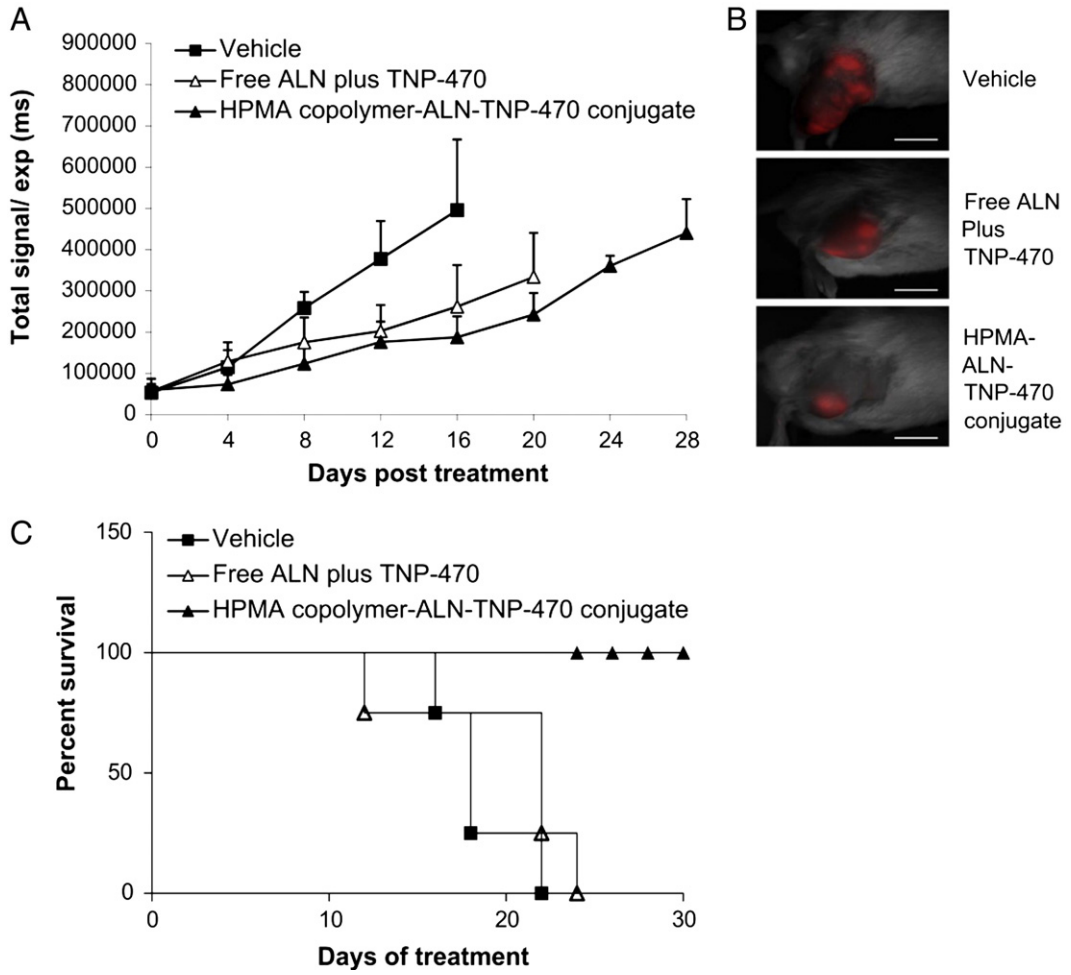

Fig. 11. HPMA copolymer-ALN-TNP-470 conjugate inhibits K7M2 murine osteosarcoma and prolong the survival rate of the mice. (A) On day 16 the conjugate (closed triangles) inhibited tumor growth by 64% ($P = 0.003$) compared with 45% ($P = 0.027$) of free ALN and TNP-470 (open triangles). Data represent mean ± s.e.m. (B) Intravital non-invasive fluorescence imaging of mCherry-labeled K7M2 tumor-bearing mice, treated with free or conjugated ALN and TNP-470 and taken on day 16 from best responses achieved. (C) Percent survival of mice treated with HPMA copolymer-ALN-TNP-470 conjugate (closed triangles), a combination of free ALN plus TNP-470 (open triangles) compared with control group (squares). Scale bar represents 10 mm (n = 5 mice per group).
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research in pediatric therapies, i.e., the PIP, promoted by governmental initiatives in the US [280–282] and Europe [283]. The creation of the Paediatric Committee's-Formulation Working Group of EMA is an interesting approach to concretely promote the development of innovative pediatric formulations and, in this scenario, the implementation of nanotechnologies to improve their performance. Another encouraging aspect during recent years was the foundation and consolidation of initiatives that comprehensively address the main challenges in the treatment of children such as the Pediatric Formulation Initiative of the Eunice Kennedy Shriver National Institute of Child Health and Human Development [284,285], the European Paediatric Formulation Initiative (EuPFI) [286] and the Global Research in Paediatrics – Network of Excellence (GRiP) [287]. These consortia devote efforts to assess and improve drug taste and palatability, a main issue in pediatric treatment [288]. The question that remains unanswered is whether these initiatives will be eager to also promote (or not) the establishment of multidisciplinary groups that will discuss the multifaceted challenges in the implementation of nanomedicines in children. According to our vision, due to market constraints, the fate of pediatric nanomedicine seems to be intimately linked to the progresses made in the implementation of these innovative therapies in adults. This is further sustained by the absence of a solid regulatory framework in nanomedicine [6], that from an ethical perspective, supports the implementation of the "Precautionary principle" what would make the implementation of nanotechnology in children a possibility only in the long-term range and after very extensive and meticulous research [28]. On the other hand, there are diseases that show especially great morbidity in children and thus they would deserve special attention and dedication beyond market considerations to assure better medicine to all. For them, the most effective response seems to be the foundation of research clusters in academia and industry that address, in a focused and multidisciplinary way, the treatment of each single disease. Otherwise, the current adult–child gap is expected to undergo a further deepening and the pediatric patients left one more time behind.

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