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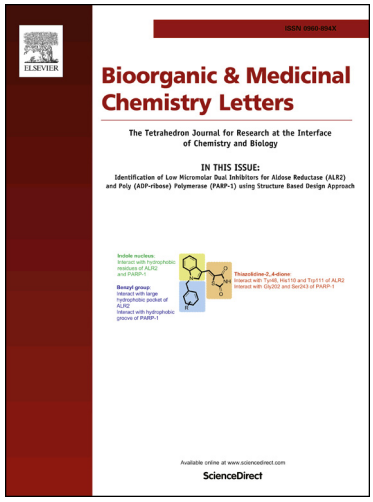

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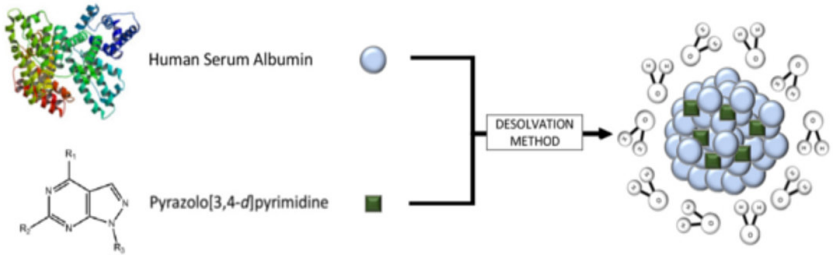

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Pyrazolo[3,4-*d*]pyrimidines-loaded human serum albumin (HSA) nanoparticles: preparation, characterization and cytotoxicity evaluation against neuroblastoma cell line

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ABSTRACT

Pyrazolo[3,4-*d*]pyrimidine derivatives **1-5**, active as c-Src inhibitors, have been selected to be formulated as drug-loaded human serum albumin (HSA) nanoparticles, with the aim of improving their solubility and pharmacokinetic properties. The present study includes the optimization of a desolvation method-based procedure for preparing HSA nanoparticles. First, characterization by HPLC-MS and Dynamic Light Scattering (DLS) showed a good entrapment efficacy, a controllable particle size (between 100 and 200 nm) and an optimal stability over time, confirmed by an *in vitro* drug release assay. Then, **1-4** and the corresponding NPs were tested for their antiproliferative activity against neuroblastoma SH-SY5Y cell line. Notably, **3-NPs** and **4-NPs** were identified as the most promising formulation showing a profitable balance of stability, small size and a similar activity compared to the free drugs in cell-based assays. In addition, albumin formulations increase the solubility of pyrazolo[3,4-*d*]pyrimidine avoiding the use of DMSO as solubilizing agent.

Neuroblastoma (NB) is the second most common extracranial malignant tumor of childhood and the most common solid tumor of infancy. It originates in the neural crest from primordial cells that physiologically evolve into adrenal medulla and sympathetic ganglion.[1] The degree of malignancy depends on the degree of cellular maturation, with the most undifferentiated and aggressive NB presenting in children (median age ≤ 2 years).[2] High-risk tumors are treated with surgery and chemotherapy, which usually includes alkylating agents (i.e. cyclophosphamide), platinum compounds, topoisomerase II inhibitors (i.e. etoposide, doxorubicin), and vincristine. Despite aggressive chemotherapy treatment, the 3-year event-free survival rate for NB patients is < 15%.[2,3] In order to improve this percentage, novel therapeutic strategies are being investigated.

c-Src is a signal-modulating non-receptor tyrosine kinase belonging to the Src-family. Its hyperactivation has been proved to be closely connected with the development and progression of several tumor types, including NB.[4,5,6] Thus, small molecules inhibitors of c-Src represent a valid approach to anticancer therapy and some of them are in clinical use for the treatment of chronic myeloid leukemia (CML) (i.e. dasatinib, bosutinib), with a reduction of the severe side effects associated with the conventional chemotherapy.[7,8] Trials are being conducted to validate their efficacy also for solid tumors (i.e. breast, prostatic and lung cancer) and results indicate promising developments.[9,10,11]

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The use of human serum albumin (HSA) nanoparticles in oncology is advantageous for many aspects. Albumin, a versatile protein carrier for drug delivery, has been shown to be non-toxic, non-immunogenic and biodegradable, and to possess several potential binding sites for drugs. A single monomer of albumin is about 7 nm in diameter and 66 kDa of molecular weight. Abumin can reversibly bind hydrophobic drugs which are transported throughout the body, improving their pharmacokinetic profile and reducing their rapid clearance.[12] In addition, albumin-loading provides tumor targeting thanks to the enhancement of the permeability and retention (EPR) effect[13] mostly due to the leak of a normal vascular structure and an efficient lymphatic drainage in the tumor mass. Moreover, it has been proposed that albumin can specifically bind to the 60 kDa glycoprotein (gp60) receptor, usually located on the endothelial cell surface and overexpressed on cell membrane of many cancer cell types. This binding promotes the formation of vesicles and results in the transcytosis and delivery of drugs into the sub-endothelial space.[14,15] Additionally tumors are sites of albumin catabolism: they utilize the protein as sustenance for their accelerated growth.[16] Taken together, all these mechanisms increase the uptake of albumin-based nanoparticles into the solid tumors, making HSA nanoparticles an interesting alternative for drug delivery in the field of anticancer therapies.[17]

A milestone in the development of new albumin-based nanoparticles has been reached with the advent on the market of *nab*-paclitaxel or Abraxane©, a paclitaxel-loaded albumin nanoparticles solvent-free formulation.[18] Abraxane© was approved in 2013 by FDA and the European Medicines Agency (EMA) and added in the list of the first-line therapy for the treatment of advanced breast cancer,[19] NSCLC[20] and pancreatic cancer[21] by the National Comprehensive Cancer Network (NCCN).

Pyrazolo[3,4-*d*]pyrimidine derivatives have been extensively studied for several years by our research group. These compounds act as ATP-competitive inhibitors of c-Src tyrosine kinase[22] and exhibit strong anti-proliferative and pro-apoptotic effects toward several cancer cell lines, including NB,[23,24] CML[25] and glioblastoma (GB) cells.[26] Pyrazolo[3,4-*d*]pyrimidine bind the catalytic site of c-Src in a manner very similar to the ATP, thus preventing the entrance of the cofactor. *In vivo* studies proved the capacity of some of these compounds to reduce tumor mass in NB xenograft mice models.[27]

Despite their promising anticancer activity, pyrazolo[3,4-*d*]pyrimidines are characterized by a sub-optimal aqueous solubility and consequently unfavorable pharmacokinetic profile that limits their potential use in cancer treatment. In the last few years, our research group spent many efforts with the aim of improving the ADME properties of such promising compounds. In a recent work the nanosystem approach for drug delivery has been applied to a small group of pyrazolo[3,4-*d*]pyrimidines selected for their promising activity against NB.[28] Stealth liposomes and HSA nanoparticles were prepared and characterized for different parameters such as the entrapment efficiency (EE%), the mean size, the polydispersity index (PDI) and ζ potential. Then, both the nanosystems have been evaluated for their antitumor efficacy on neuroblastoma SH-SY5Y cell line viability. As a result, albumin nanoparticles, prepared by disulphide-bond induced self-assembly method, gave no positive outcomes, exhibiting limited drug loading, large mean diameter, broad size distribution, instability and tendency to form aggregates.

In this study, on the basis of their inhibitory activity on c-Src (K_i in the submicromolar range), five pyrazolo[3,4-*d*]pyrimidines have been selected from among our extensive library to be formulated as albumin nanoparticles (Figure 1).[24,27,29] For this purpose, the coacervation/desolvation method has been used and optimized for our compounds in order to improve the characterization parameters of our previous formulation.[28] The main objective was to obtain reproducible nanoparticles of small diameter (<200 nm) and negative charge surface able to avoid *in vivo* opsonisation and enhance blood circulating time.[30]

The protocol, already proposed by Weber *et al.* (2000),[31] has been adopted and optimized in some points for the loading of our molecules. Specifically, 50 mg of HSA were dissolved in 0.5 mL of 10 mM NaCl solution, pH 9; under constant stirring (500 rpm) at room temperature, desolvation of the 10% HSA solution was executed using a syringe pump (KD Scientific Syringe Pump) by addition of 2 mL of aqueous 75% p/p acetone solution, in which the compound was previously dissolved. Then an aqueous 8% glutaraldehyde solution was dropwise added for cross-linking and stabilizing the particles.

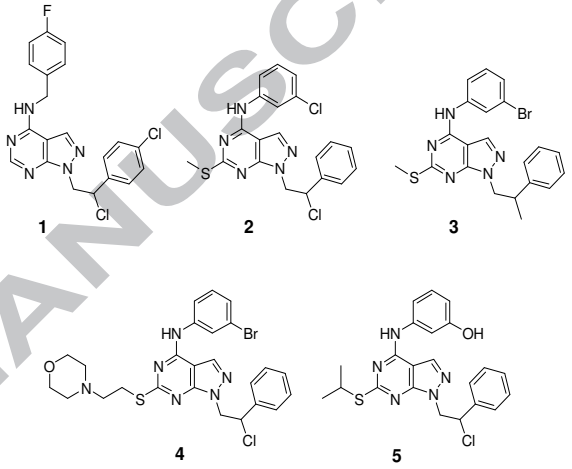

Figure 1. Molecular structure of compounds **1-5**.

The suspension was left to stir at room temperature for 16 h. Acetone was evaporated under reduced pressure; non-encapsulated compound and the excess glutaraldehyde were removed by three repeated cycles of ultracentrifugation at 20000 g for 30 minutes each (BeckMan Coulter Optima L-90K). Washing waters were collected to perform Bradford's assay for the determination of the amount of free albumin. The residue was lyophilized at -70°C (Freeze Dry LIO5P) for 48 hours using a cryoprotectant 3% p/v sucrose solution. All compounds were loaded using the same protocol.

The amount of albumin forming nanoparticles was determined by the difference between the total amount of initial protein added and the amount of protein in the supernatants obtained during purification step. Bradford assay was used for quantitative determination of HSA.[32] The lyophilized final sample was suspended in acetonitrile to achieve the denaturation of albumin and centrifuged at 4500 rpm for 15 minutes. The concentration of the compound in the supernatant solution was determined by HPLC-UV-MS (HPLC-UV-MS methods in the supporting material). Results were compared with the standard curve and calculated with the following equations:

Eq. 1 $$\%\ \mathrm{EE} = \frac{\text{amount of drug in supernatant}}{\text{amount of total drug}} * 100$$

Eq. 2 $$\text{Drug Load} = \frac{\text{amount of drug in supernatant}}{\text{HSA weight}}$$

Pyrazolo[3,4-*d*]pyrimidines-carried nanoparticles, prepared by the desolvation method, showed an excellent entrapment efficacy % (EE%) with the best results for **4-NPs** and **3-NPs** (99.5%) and the worst EE%, but still satisfying, of 40% for **1-NPs**. The EE% values showed that the encapsulation rate in albumin nanoparticles is independent from the aqueous solubility. Indeed, compounds **3** and **4** presented the same highest EE% even if they exhibit the lowest and the highest solubility, respectively, as shown in Table 1.

Mean particle size, size distribution and ζ-potential were determined by Dynamic Light Scattering (DLS) (Zeta Sizer Nano ZS90, Malvern Instruments Ltd, Malvern, UK). The lyophilized nanoparticles were dispersed in water and measured at 24°C with a scattering angle of 90°. To determine the stability of this formulation stored at 4°C, the size and ζ-potential were monitored up to 7, 15 and 30 days (values reported in Table S1).

Table 1. In vitro activity against Src and ADME properties[a], nanoparticles drug loading and encapsulation efficacy % **1-5**

Cpd	Src (Ki μM)	P app[b] (10-6cm/sec 50% DMSO)	Aq. Solub.[c] (μg mL^{-1})	Met. Stab.%[d]	HSA NPs	
					Drug Loading (μg/mg)	EE%
1	0.8	16.6	<0.04	94.0	39.12	40.0
2	0.72	0.26	0.126	91.1	62.66	64.0
3	0.02	0.01	<0.01	95.5	94.81	99.5
4	0.13	5.27	3.71	96	90.87	99.5
5	0.01	4.53	0.016	93.5	53.88	55.5

[a]All data were previously reported.[24,27,33] [b]Apparent permeability was determined with PAMPA assay. [c]Aqueous solubility was determined by means of the LC-UV method. [d]The human liver microsomal stability is expressed as percentage of unmodified parent drug.

Particle diameter should be in the sub-150 nm range with narrow size distribution to reduce *in vivo* opsonisation and avoid their removal by simple filtration in a capillary bed after intravenous injection.[34] ζ-potential is a stability indicator and offers prediction about the tendency of nanoparticles to form aggregates over time. According to DLS measurements, freshly prepared nanoparticles presented diameters ranging from 49.8 nm (**5-NPs**) to 114.6 nm (**3-NPs**). For all the samples, ζ-potential was around -30 mV, falling within the stability range (values greater than +25 mV or less than -25mV are considered to be ideal).[35] With time, HSA nanoparticles slightly tended to increase their dimensions. Nevertheless, only 5-NPs became larger than 150 nm after 30 days and showed the presence of two different particle populations, confirming to be the less stable formulation. **4-NPs**, presenting high EE% and a better water solubility compared with the other NPs was chosen to determine the drug release profile *in vitro* in physiological conditions (PBS with bovine serum albumin 50 mg/mL at pH 7.4 at 37°C under constant stirring). The cumulative drug release percentage was measured over a 96 h-period. The results exhibited a slow monophasic release of **4** from its formulation **4-NPs**, considering that only the 7 % of the compound was released in the first 6 hours, 21 % after 24 h and 44.6 % after 96 hours. The continue, progressive and sustained release of pyrazolo[3,4-*d*]pyrimidines from NPs is due to the slow diffusion of drug molecules through the pores of the cross-linked albumin matrix. This release profile confirms the stability of our formulation in physiological settings and validates its use *in vivo* as controlled release drug delivery system allowing longer circulating half-life and continuous targeting of the tumor cells.[36]

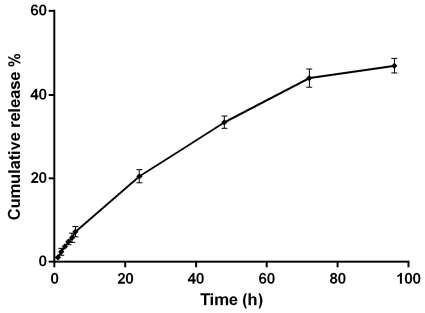

Figure 2. Release profile of **4-NPs** in physiological conditions.

Finally, free **1-4** dissolved in DMSO, **1-4NPs** and empty NPs suspended in saline solution were evaluated for their cytotoxic effect on SHSY-5Y NB cells.

5-NPs was excluded because of instability of mean size and size distribution over time. Cells were treated for 48 h with increasing concentrations of compounds (0,1-100 μM) and their cytotoxicity was calculated considering the number of viable cells in respect to the control (Figure 3).

Notably, the activities of loaded NPs demonstrated to be dependent from the EE% (Table 1). Indeed, the higher difference in activity was observed for compound **1** that presented an entrapment efficiency rate of 40%. The free molecules showed an IC_{50} value of 2.83 μM while **1-NPs** demonstrated a lower cytotoxic effect with an IC_{50} of 19.37 μM. The observed difference could be attributed to the lower concentration of molecule included into the NPs per single carrier and non-complete release of the free drug.

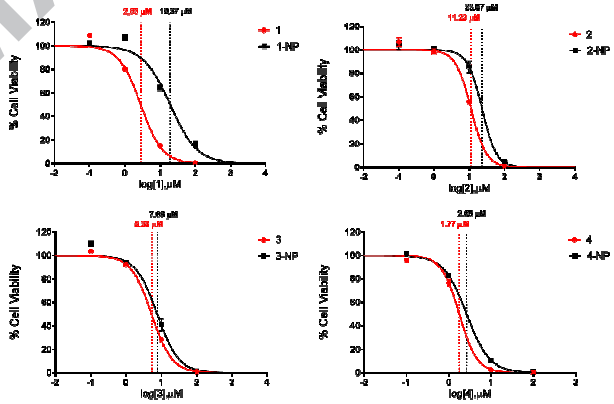

Figure 3. Viability of SH-SY5Y human NB cell line treated for 48h with free **1-4** (black curves) and **1-4NPs** (red curves).

The same trend was observed for compounds **2-4** and **2-4NPs**: **3-NPs** and **4-NPs** for which the entrapment efficiency was about 99% had a cytotoxic effect on neuroblastoma SH-SY5Y cell line comparable to their free drug (Table 2), while **2-NPs**, with an encapsulation rate of 64% still demonstrate a lower activity compared to the free **2**. For further demonstrating that the cytotoxic effect of HSA-NPs was due to drug release and not from formulation itself, empty albumin nanoparticles were tested and demonstrated to be not cytotoxic (Figure S5). Only at high concentration empty NPs become moderately cytotoxic probably because of the increased presence of glutaraldehyde cross-linked to albumin.

Table 2. Comparison of IC_{50} between free compounds and compounds-loaded nanoparticles obtained from SH-SY5Y cells viability

	48 h Treatment	
Cpd	**IC_{50} free drug (μM)**	**IC_{50} NPs (μM)**
1	2.83	19.37
2	11.23	23.07
3	5.32	7.68
4	1.77	2.65

In conclusion, we have herein reported the preparation of pyrazolo[3,4-*d*]pyrimidines-loaded albumin nanoparticles by desolvation method obtaining a significant improvement in comparison to our previous nanoparticles formulation prepared by disulphide-bond induced self-assembly method.[28] We obtained an excellent EE% (between 99.5% and 40%), a good drug loading capacity (from 39.12 to 94.81 μg of drug/mg of HSA), optimal NPs' diameter ranging from 49.8 nm to 114.6 nm and ζ-potential values equal or less than -29.9 mV for each nanoparticles formulation. Repeated DLS analysis confirmed the stability over time of our albumin nanoparticles systems. The *in vitro* drug release curve, characterised by a slow and constant growth, was a further evidence of their stability. Moreover, cytotoxicity of **3-NPs** and **4-NPs** on SHSY-5Y cell line was shown to be comparable to the effect of free drugs, demonstrating the validity of the albumin nanoparticles as pyrazolo[3,4-*d*]pyrimidines-carrier system. Finally, albumin NPs can be dissolved in physiological solution avoiding the use of DMSO or other organic solvent for biological evaluations, both *in vitro* and *in vivo*. The increase in solubility and stability of the NPs encapsulated drugs will presumably result in more favourable and suitable pharmacokinetic properties (longer circulating half-life, increased bioavailability, tumor accumulation). Taken together, our results of characterization and *in vitro* anticancer activity of pyrazolo[3,4-*d*]pyrimidines-HSA NPs are promising and open the way for future *in vivo* experiments.

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Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.