Heteronanoparticles by Self-Assembly of Ecdysteroid and Doxorubicin Conjugates To Overcome Cancer Resistance

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Supporting Information

ABSTRACT: Heteronanoparticles (H-NPs) consisting of conjugates characterized by a squalene tail linked to doxorubicin and ecdysteroid derivatives are presented. Biological evaluation on A2780ADR cell line confirms not only the maintenance of the activity of the parental drug but also the ability to overcome cancer resistance. The *in vitro* cell uptake was demonstrated, and the involvement of an endosomal-mediated pathway was suggested.

KEYWORDS: Self-assembled-nanoparticles, ecdysteroids, doxorubicin, drug toxicity, cancer

The formation of self-assembled nanoparticles (NPs) using anticancer drug conjugates could be a useful and smart approach to face cancer.^{1–3} In the past few years, we developed a step-by-step project that moved from the preparation of a novel class of squalene conjugates with paclitaxel, podophylotoxin, camptothecin, and epothilone A⁴ and reached, as highest evidence of efficacy, the preparation of heteronanoparticles with doxorubicin and cyclopamine conjugates that were able to reduce *in vivo* the tumor growth and toxicity due to the use of the single drugs.^{5–8} The conjugates were characterized by a squalene tail that makes them able to self-assemble in water and by a drug unit connected via a disulfide-containing linker to secure the release inside the cell.

Our interest in facing the resistance of different kinds of tumor cells⁹ drove us to consider the formation of self-assembled heteronanoparticles as a promising approach. Martins et al. recently reported the chemo-sensitizing effect

of apolar ecdysteroids on MDR cancer cells.¹⁰ The anti-proliferative activity of these compounds was very low, and they exerted a mild activity in modulating the ABCB1-mediated efflux of rhodamine 123. Further studies revealed that the chemo-sensitizing activity can be independent of efflux inhibition, in particular, ketals of poststerone, a known *in vivo* metabolite of 20-hydroxyecdysone that sensitized ABCB1-transfected cancer cells to doxorubicin in a highly MDR selective manner without significantly interfering with the efflux function.

In this scenario, we planned the preparation of some ecdysone conjugates containing the squalene tail to be combined with the known squalenoylated doxorubicin¹¹

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(Chart 1) and investigate their ability to form heteronano-particles (H-NPs) toward the treatment of doxorubicin

Chart 1. Schematic Representation of the Building Blocks and Obtained Conjugates

resistant cell lines. In the light of the synergistic effect of ecdysteroid acetonides with doxorubicin,¹⁰ we focused our attention on diketals of 20-hydroxyecdysone and related derivatives of poststerone.

Five different conjugates were prepared according to Schemes 1 and 2 (see Table 1 and also the Supporting

Scheme 1. Synthesis of the Conjugates 3a–c

Scheme 2. Synthesis of the Conjugates 6a,b

Table 1. Ecdysteroid Conjugates and Corresponding Starting Material

starting material	X	PG	deprotection	product
1a + 2a	CH ₂	Bn	H ₂ , Pd(OH) ₂	3a
1b + 2a	CH ₂	Bn	H ₂ , Pd(OH) ₂	3b
1a + 2b	S	(CH ₂) ₂ SiMe ₃	TBAF	3c
4 + 5a	CH ₂			6a
4 + 5b	S			6b

Information). 20-Hydroxyecdysone derivatives 3a–c were synthesized following a three-step procedure (Scheme 1): (a) condensation reaction between the protected 20-hydroxyecdysone 1 and the proper acid 2, (b) deprotection of the carboxylic group, and (c) final condensation with 1',2-tris-norsqualene alcohol. Compounds 6a and 6b were prepared by a condensation reaction between compounds 5⁴ and 4.¹²

Next, the ability of the conjugates to self-assemble in nanoparticles was investigated. Briefly, compound solution in THF was added dropwise into ultrapure water. The spontaneous self-assembly into NPs is a consequence of local interactions between the hydrophobic molecules, mainly guided by squalene chains interactions. Subsequently, the organic solvent was removed under reduced pressure (see Supporting Information). The nanosuspensions were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). DLS analysis confirmed the formation of nanoassemblies in aqueous medium. The assemblies were monodisperse (PDI < 0.2) with sizes around 200 nm, except for the 6a NPs that exhibited a 2-fold larger hydrodynamic diameter (366.3 ± 20.17 nm) (Table 2). The highly negative ζ -potential values (< -20.0 mV) suggested that the electrostatic repulsion between NPs contributed to the stability of the nanoassemblies in aqueous medium.

Table 2. NPs Characterization by Dynamic Light Scattering (ZetaSizer, Malvern)^a

NPs	h.d. $\pm$ SD (nm)	P.I.	ζ -pot. $\pm$ SD (mV)
3a	198.1 $\pm$ 0.9	0.106 $\pm$ 0.041	-49.8 $\pm$ 0.9
3b	205.1 $\pm$ 0.6	0.050 $\pm$ 0.026	-43.0 $\pm$ 0.9
3c	214 $\pm$ 3.4	0.086 $\pm$ 0.046	-30.0 $\pm$ 4.6
6a	366.3 $\pm$ 20.17	0.161 $\pm$ 0.032	-21.5 $\pm$ 4.55
6b	221.8 $\pm$ 4.879	0.081 $\pm$ 0.088	-21.7 $\pm$ 1.50

^ah.d., hydrodynamic diameter; P.I., polydispersity index; ζ -pot., ζ -potential.

Compounds 3a–c and 6a,b were further mixed with squalene-conjugated doxorubicine (DOXO-Sq) that is well-known to have a better therapeutic index than doxorubicin¹¹ and to form heteronanoparticles (H-NPs) according to our recent paper.⁷ H-NPs were prepared by mixing the compound solutions (125 μ L, 250 μ g, 2 mg mL⁻¹) with DOXO-Sq solution (2 mg mL⁻¹) in THF with a molar ratio compound/DOXO-Sq of 50 and by dropping the organic solution into 250 μ L of ultrapure water as previously described.¹¹ The mixture became opalescent suggesting the possible formation of particles. H-NPs filtered with a 0.45 μ m filter were characterized by DLS and TEM. DLS analysis (Table 3)

Table 3. H-NPs Characterization by Dynamic Light Scattering (ZetaSizer, Malvern)^a

X:DOXO-Sq 50:1			
X	h.d. $\pm$ SD (nm)	P.I.	ζ -pot. $\pm$ SD (mV)
3a	533.1 $\pm$ 26.60	0.160 $\pm$ 0.104	-9.73 $\pm$ 0.95
3b	538.2 $\pm$ 33.41	n.d.	-9.39 $\pm$ 2.91
3c	579.2 $\pm$ 104.4	0.226 $\pm$ 0.276	-20.3 $\pm$ 2.52
6a	187.7 $\pm$ 14.48	0.223 $\pm$ 0.069	-13.5 $\pm$ 9.65
6b	298.7 $\pm$ 11.43	0.264 $\pm$ 0.014	-11.1 $\pm$ 3.48

^ah.d., hydrodynamic diameter; P.I., polydispersity index; ζ -pot., ζ -potential.

confirmed the formation of H-NPs in aqueous medium. The assemblies were monodisperse (PDI < 0.2) but larger compared to the homogeneous NPs, except for the 6a,b NPs where DOXO-Sq resulted in a decrease of (or did not alter) the particle dimension, respectively. Also, the ζ -potential values decreased.

Figure 1 reports TEM analysis for NPs obtained by self-assembly of compound **6b** and the H-NPs **6b**/DOXO-Sq

Figure 1. TEM analysis for NPs-**6b** (a, scale reported 1 μm) and H-NPs **6b**/DOXO-Sq (b, scale reported 500 nm).

(50:1). The images show compact nanoparticles whose diameter is smaller if compared with the data obtained by DLS but similar in the case of H-NPs.

The ability of both NPs and H-NPs to inhibit cell growth was assayed on A2780ADR cells, a human ovarian carcinoma doxorubicin-resistant cell line. The obtained results are shown in Table 4 and are expressed as GI_{50} values, i.e., the concentration of the test agent inducing 50% reduction in cell number compared with control cultures.

Table 4. Inhibition of A2780ADR Cell Growth

	A2780-ADR cells (GI_{50} μM) ^a	
	NPs	X:DOXO-Sq 50:1
3a	>50	0.50 ± 0.02
3b	>50	0.78 ± 0.02
3c	>50	0.69 ± 0.15
6a	19 ± 3	0.34 ± 0.08
6b	39 ± 4	0.26 ± 0.03
DOXO-Sq	1.17 ± 0.06	---

^aValues are the mean $\pm$ SD of at least four independent experiments.

Considering the NPs, it can be noted that DOXO-Sq exerts an interesting inhibitory effect on A2780ADR proliferation, showing a GI_{50} value of about 1 μM . This capacity, notwithstanding about ten times lower with respect to that of doxorubicin ($\text{GI}_{50} = 0.16 \pm 0.01 \mu\text{M}$), confirms, in accordance with previous studies,¹¹ the ability of the drug to induce an antiproliferative effect even following squalenoylation. Otherwise, the NPs obtained by squalene-conjugated ecdysteroids provoke in the same experimental conditions a negligible effect on cells, and indeed, only for the nanoassemblies **6a** and **6b** a detectable and quite low cytotoxicity on A2780ADR cells was demonstrated. Notably, the H-NPs obtained by mixing **3a–c** and **6a,b** with DOXO-Sq induce a significant antiproliferative effect, with GI_{50} values from 1.5 to more than 4 times lower with respect to that obtained for DOXO-Sq. These data suggest the ability of the H-NPs to overcome, at least partially, the resistance phenomenon. In this connection, it was of interest to investigate the effect of tamoxifen, a well-known inhibitor of the P-glycoprotein¹³ on the intracellular uptake of the H-NPs **3a**/DOXO-Sq, taken as an example, and of doxorubicin, used as reference. The cell internalization in A2780ADR was monitored by flow cytometry. As expected, the presence of tamoxifen significantly increased the drug accumulation in these cells. In detail, the intracellular mean fluorescence intensity of doxorubicin rose by 27% after exposure to the P-glycoprotein

inhibitor. Otherwise, no difference in fluorescence was observed between the cells treated and those not treated with tamoxifen. This result further supports the hypothesis that these new H-NPs can play a crucial role in drug resistance mechanism, suggesting the inability or at least the reduction of P-glycoprotein to mediate their efflux from resistant cells. The *in vitro* cell internalization was then further investigated by fluorescence microscopy to verify any change in the uptake pathway. For this purpose, A2780ADR cells were incubated for 2 h in the presence of doxorubicin and the H-NPs **3a**/DOXO-Sq. The results are shown in Figure 2a,b, respectively.

Figure 2. Fluorescence microscopy of A2780ADR cells incubated for 2 h in the presence of doxorubicin (a) or H-NPs **3a**/DOXO-Sq (b) at 5 μM . The nucleus and cytoplasm were stained with DAPI and antibody antitubulin conjugated with AlexaFluor488, respectively.

The cell penetration of both agents is rapid; nevertheless, the intracellular localization differs: for the drug, a major localization in the nuclei is observed (a), while for the H-NPs, a staining almost exclusive in the cytoplasm appears (b).

A more in depth microscopy analysis (Figure 3) highlights in the cytoplasm of A2780ADR cells treated with the H-NPs **3a**/

Figure 3. Fluorescence microscopy of A2780ADR cell treated for 2 h with H-NPs **3a**/DOXO-Sq.

DOXO-Sq the occurrence of vesicles characterized by a mean diameter of about 0.9 μm , a value significantly higher with respect to the hydrodynamic diameter of the corresponding H-NP (533.1 ± 26.60 , Table 3). The more complex membrane trafficking pathway of NPs¹⁴ with respect to free doxorubicin¹⁵ could be responsible for the delay in nucleus accumulation of the H-NPs **3a**/DOXO-Sq.

The obtained results highlight the effectiveness of self-assembled H-NPs to face cancer resistance and show a general

strategy that could be applied in various pathologies where combined therapy could be beneficial.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00078.

Experimental details regarding preparation of compounds 3a–c and 6a,b, nanoparticles preparation and characterization, and biological evaluation (cell cultures, inhibition growth assay, cytofluorimetric analysis, and confocal microscopy analysis) (PDF)

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Notes

The authors declare no competing financial interest.

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