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

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# Hetero-Nanoparticles by self-assembly of ecdysteroid and doxorubicin conjugates as promising approach to overcome cancer resistance

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*KEYWORDS Self-assembled-Nanoparticles, Ecdysteroids, Doxorubicin, Drug toxicity, Cancer*

**ABSTRACT:** Hetero-nanoparticles (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.

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 last few years, we developed a step-by-step project that moved from the preparation of a novel class of squalene-conjugates with paclitaxel, podophyllotoxin, camptothecin and epothilone A[4] and reached, as highest evidence of efficacy, the preparation of hetero-nanoparticles with doxorubicin and cyclopamine conjugates that were able to reduce *in vivo* the tumour 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 kind of tumor cells,[9] drove us to consider the formation of self-assembled hetero nanoparticles as a promising approach. Martins *et al.* recently reported the chemo-sensitizing effect of apolar ecdysteroids on MDR cancer cells.[10] The antiproliferative 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, 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 doxorubicin[12] (Chart 1) and investigate their ability to form hetero-nanoparticles (H-NPs) towards the treatment of doxorubicin 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.

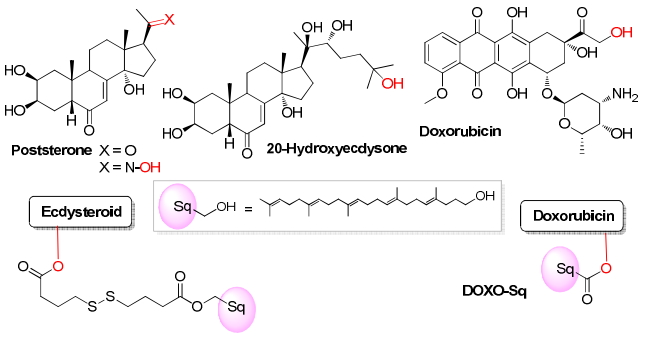
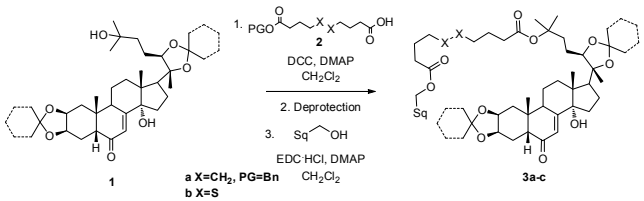
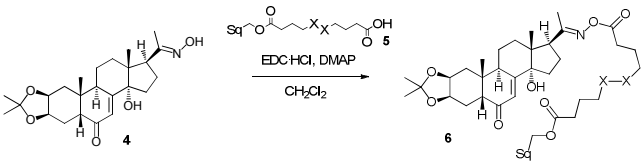



**Chart 1.** Schematic representation of the building blocks and obtained conjugates.

Five different conjugates were prepared according to Schemes 1 and 2 (see Supp. Info.). 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 **5**[4] and **4**.[11]




**Scheme 1.** Synthesis of the conjugates **3a-c**.




**Scheme 2.** Synthesis of the conjugates **6a,b**.

| Starting material | X | PG | Deprotection | Product |
|---|---|---|---|---|
| **1**-diacetonide | $CH_2$ | Bn | $H_2$, $Pd(OH)_2$ | **3a** |
| **1**-di(cyclohexylacetal) | $CH_2$ | Bn | $H_2$, $Pd(OH)_2$ | **3b** |
| **1**-diacetonide | S | $(CH_2)_2SiMe_3$ | TBAF | **3c** |
| **4** | $CH_2$ | - | - | **6a** |
| **4** | S | - | - | **6b** |

**Table 1.** Ecdysteroid conjugates and corresponding starting material.

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 Supp. Info.). 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 twofold larger hydrodynamic diameter (366.3 nm ± 20.17 nm). 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*.

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

**Table 2.** NPs characterization by dynamic light scattering (ZetaSizer, Malvern). *h.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 doxorubicin[12] and to form hetero-nanoparticles (H-NPs) according to our recent paper.[7] H-NPs were prepared by mixing the compound solutions (125 µl, 250 µg, 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 µl of ultrapure water as previously described. The mixture became opalescent suggesting the possible formation of particles. H-NPs filtered with a 0.45µm filter were characterized by DLS and TEM. DLS analysis (Table 3) 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.

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

**Table 3.** H-NPs characterization by dynamic light scattering (ZetaSizer, Malvern). h.d.: hydrodinamic diameter, P.I.: Polydispersity Index; ζ-pot.: ζ-potential.

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

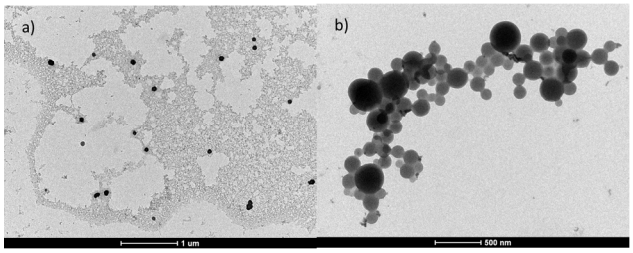



**Figure 1.** TEM analysis for NPs-**6b** (a, scale reported 1 µm) and H-NPs **6b/DOXO-Sq** (b, scale reported 500 nm)

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.

| | 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 | ---- |

**Table 4.** Inhibition of A2780ADR cell growth.. [a]Values are the mean ± 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 ($GI_{50}$= 0.16 ± 0.01 μM) confirms, in accordance with previous studies,[12] 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-glycoprotein[13] 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 two hours in the presence of doxorubicin and the H-NPs **3a/DOXO-Sq**. The results are shown in Figure 2a and 2b, respectively.

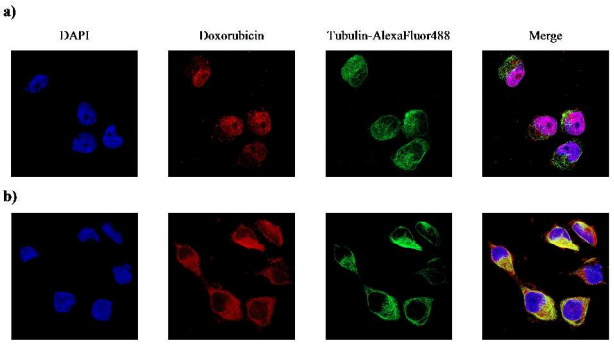



**Figure 2**. Fluorescence microscopy of A2780ADR cells incubated for 2h in the presence of doxorubicin (a) or H-NPs **3a/DOXO-Sq** (b) at 5 μM. The nucleus and cytoplasm were stained with DAPI and antibody anti-tubulin conjugated with AlexaFluor488, respectively.

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/DOXO-Sq**, the occurrence of vesicles characterised by a mean diameter of about 0.9 μm, a value significantly higher with respect to the hydrodynamic diameter of the corresponding H-NP (538.2 ± 33.41, Table 3). Such observation suggests that the H-NPs could enter into cell through an endosome-mediated pathway, a mechanism already demonstrated for **DOXO-Sq** in a human pancreatic cell line.[12]

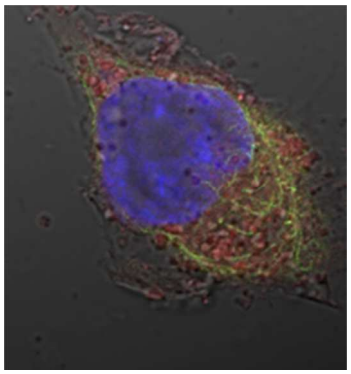


**Figure 3.** Fluorescence microscopy of A2780ADR cell treated for 2h with H-NPs **3a/DOXO-Sq**..

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. It contains the experimental details regarding: preparation of compounds **3a-c** and **6a**,**b**, nanoparticles preparation and characterization, biological evaluation. (cell cultures, inhibition growth assay, cytofluorimetric analysis, confocal microscopy analysis)

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. *These authors contributed equally.

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