

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

## Heteronanoparticles by self-Assembly of Doxorubicin and Cyclopamine Conjugates

### **This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1654873> since 2017-12-18T14:07:58Z

*Published version:*

DOI:10.1021/acsmchemlett.7b00262

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

## Supporting Information

### Nanoparticles

- *Preparation*
- *Characterization*

### *In vitro* evaluation

- *Cell cultures*
- *Cytotoxicity assay*
- *Proliferation and apoptosis*
- *Confocal microscopy*

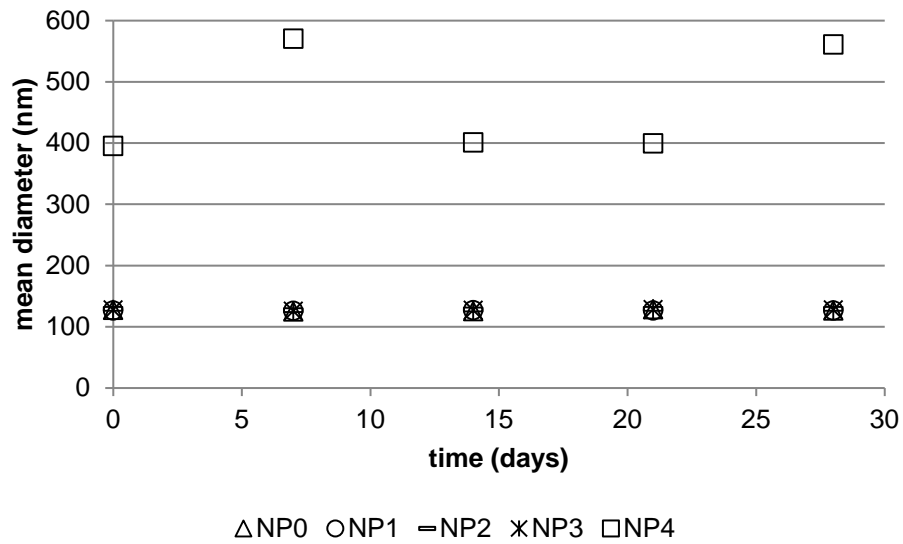
### *In vivo* evaluation

- *Xenograft experiments*
- *Immunofluorescence*

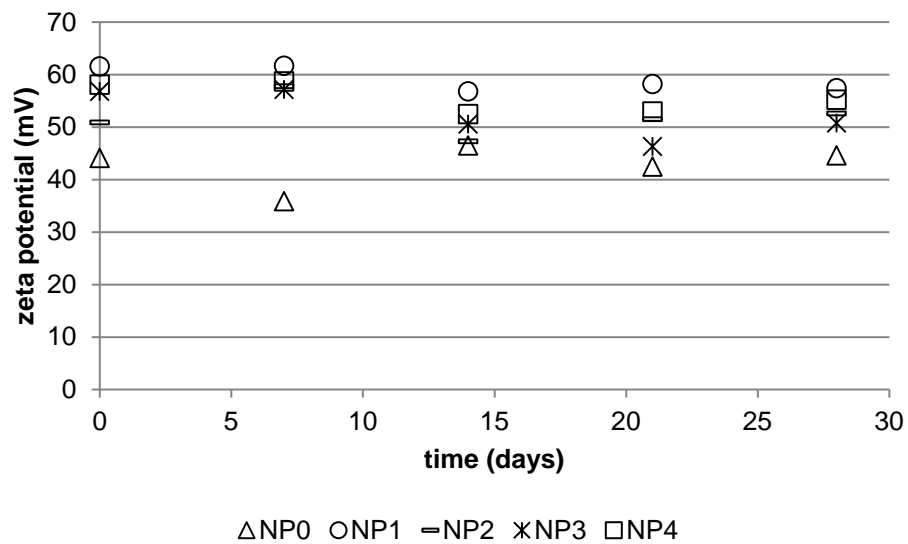
**Preparation of nanoparticles.** Cyp-SQ (**1**),<sup>6</sup> DOXO-SQ (**2**)<sup>7</sup> and Cyp-SQ/DOXO-SQ (**1:2**) NPs were prepared by nanoprecipitation. Practically, for Cyp-SQ NPs 2 mg of **1** were dissolved in THF; the organic solution was then added dropwise under magnetic stirring (500 rpm) into a double volume of MilliQ grade water. Formation of the NPs occurred spontaneously without using any surfactant. Finally, the organic solvent was completely evaporated under vacuum and an aqueous suspension of Cyp-SQ NPs was obtained (final Cyp-SQ concentration: 2 mg/ml). For DOXO-SQ NPs the same procedure was followed, except that the organic solution was poured into a double volume of PBS 1 mM pH 7.4 instead of MilliQ water (final DOXO-SQ concentration: 2 mg/ml). For hetero-NPs, **1** and **2** were codissolved in THF in various Cyp-SQ/DOXO-SQ molar ratios (1:1 (**NP1**), 5:1 (**NP2**), 10:1 (**NP3**)) with a constant Cyp-SQ amount (2 mg). Then, the THF solution of the squalene conjugates was poured into MilliQ water and the organic solvent was evaporated (final Cyp-SQ concentration: 2 mg/ml). The aqueous suspensions of the NPs were stored at 4°C in the dark.

**Characterization of nanoparticles.** The average particle size and the polydispersity index of all formulations were determined by dynamic light scattering (DLS) with a Nano ZS zetasizer (Malvern, UK) at a fixed angle of 173° and at a temperature of 25°C. The NPs dispersions were diluted 1:20 v/v with MilliQ water before analysis. Each value reported is the average of five measurements. To determine the zeta potential, the NPs samples were diluted 1:20 v/v with MilliQ water and placed in the electrophoretic cell of the Nano ZS zetasizer at 25°C. Each sample was analyzed in triplicate. The colloidal stability of the suspensions was

evaluated by measuring the mean size and the zeta potential of the NPs over a storage period of 28 days at 4°C. For Cyp-SQ/DOXO-SQ NPs 1:1 (NP1) the stability of the formulation was also tested after dilution 1:10 v/v in PBS 10 mM pH 7.4 and in fetal bovine serum (FBS, Lonza).



**Supplementary Figure 1.** Mean diameter of the different NP formulations as a function of time over a storage period of 28 days at 4°C.



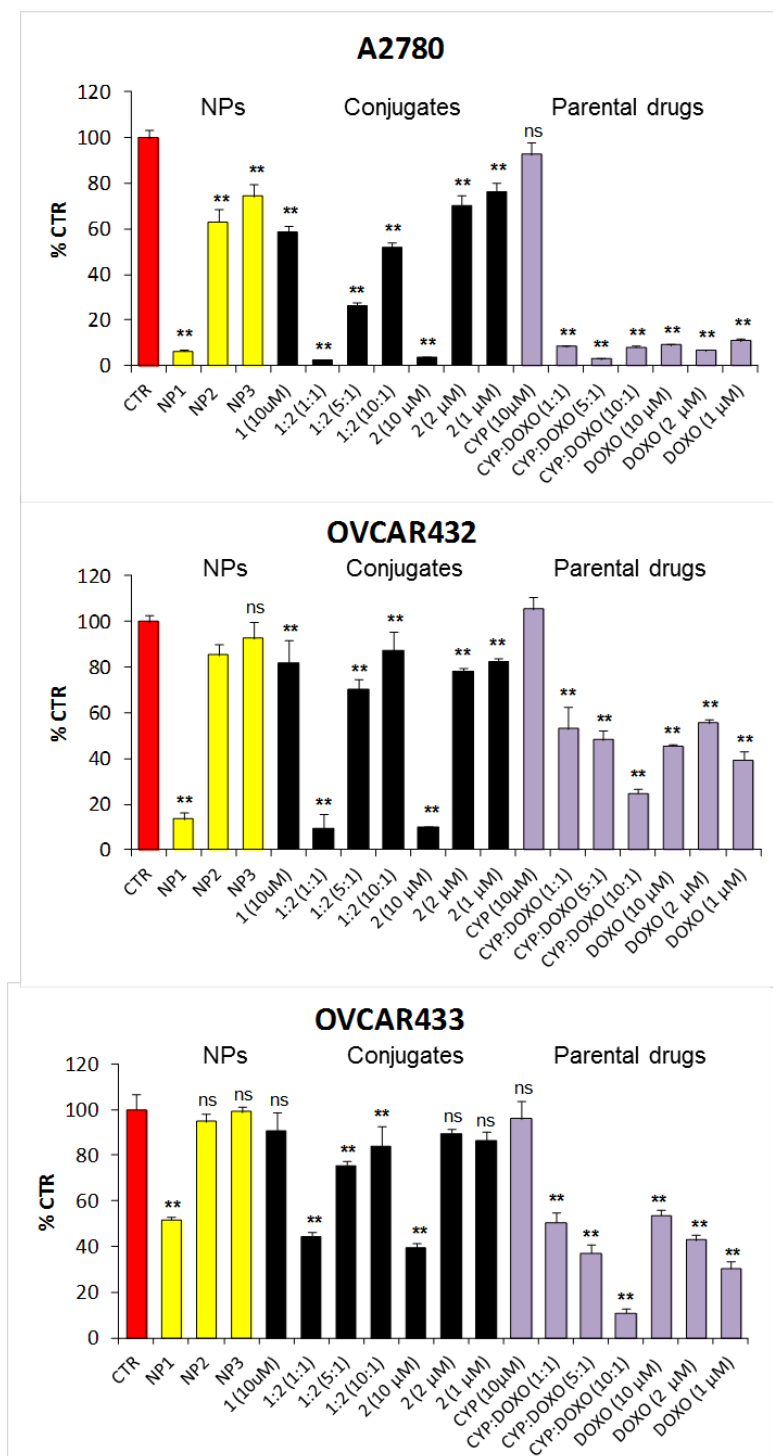
**Supplementary Figure 2.** Zeta potential of the different NP formulations as a function of time over a storage period of 28 days at 4°C.

### ***In vitro* evaluation on different cell cultures**

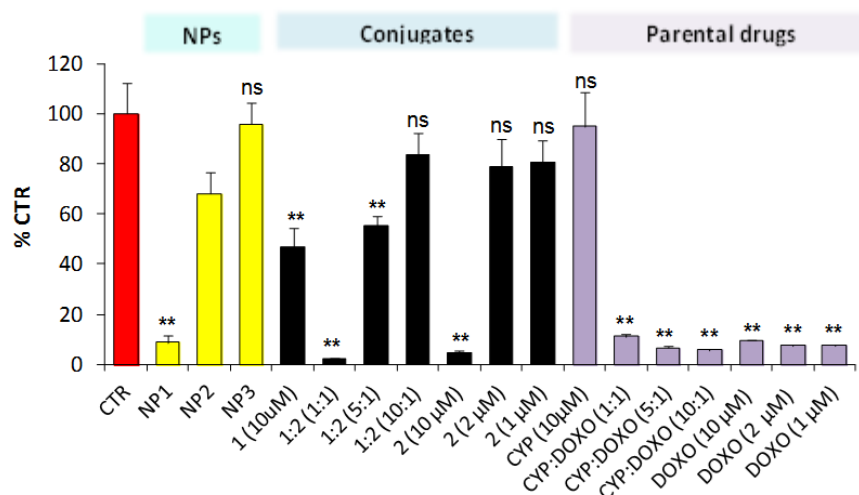
**Cell cultures.** Ovarian cancer cell lines A2780, IGROV, OVCAR432 and OVCAR433 were cultured in RPMI1640 (Lonza), with the addition of 10% FBS and 2mM glutamine (Lonza). The human epidermoid carcinoma cell line A431 was cultured in EMEM medium (Sigma-Aldrich), supplemented with 2 mM L-glutamine (Gibco), 10% FBS, 1% non essential aminoacids (Gibco) and penicillin/streptomycin (Gibco). The cells were cultured at 37°C in a moist atmosphere of 5% carbon dioxide in air.

**Cytotoxicity assay.** For cytotoxic experiments, cells were seeded at 20000 cells/mL in 96 well-plates (Corning). After 48 h cells were treated with single drugs (DOXO at 10, 2, and 1  $\mu$ M and CYP at 10  $\mu$ M) alone and in combination (at ratios CYP:DOXO, 1:1, 5:1, and 10:1), with drug derivatives (DOXO-SQ, and CYP-SQ alone and in combination at the same doses and ratios of <sup>3</sup> single drugs), and with nanoparticles (NPs). After 72 h of treatment, cell viability was assessed by MTS assay, and calculated on 5 replicates as %Ctr (mean Abs treated sample/mean Abs control sample\*100)  $\pm$ standard deviation.

**Proliferation and apoptosis.** A431 cells were seeded at 20000 cells/well in 24-well plates, or 40000/well in 6-well plates. After 48 h, the culture medium was changed with fresh medium containing the indicated drugs. For assessing apoptosis, cells were harvested 48 h after drug addition. For proliferation assays, 47 h after drug administration, BrdU was added to the culture medium at 10  $\mu$ M. Cells were harvested 1 h later. Proliferation and apoptosis were detected using the Apoptosis, DNA damage and Cell Proliferation kit from BD Biosciences, according to the manufacturer's instructions. FACS analysis was performed using LSR Fortessa and FACS DiVa software (BD Biosciences).



**Supplementary Figure 3.** Cytotoxic activity in ovarian cancer cell lines (A2780, OVCAR432, and OVCAR433) of cyclophosphamide, doxorubicin (reported as Parental drugs), compounds 1 and 2 not nanoprecipitated (reported as Conjugates) and nanoprecipitated compounds 1 and 2 (reported as NP). Concentration of the samples: NP1 (nanoprecipitated CYP-SQ = DOXO-SQ = 10  $\mu$ M); NP2 (nanoprecipitated CYP-SQ = 10  $\mu$ M, DOXO-SQ = 2  $\mu$ M; NP3 (nanoprecipitated CYP-SQ = 10  $\mu$ M, DOXO-SQ = 1  $\mu$ M; 1:2 (1:1): CYP-SQ = DOXO-SQ = 10  $\mu$ M; 1:2 (5:1): CYP-SQ = 10  $\mu$ M, DOXO-SQ = 2  $\mu$ M; 1:2 (10:1): CYP-SQ = 10  $\mu$ M, DOXO-SQ = 1  $\mu$ M; CYP:DOXO (1:1): CYP = DOXO = 10  $\mu$ M, CYP:DOXO (5:1): CYP = 10  $\mu$ M, DOXO = 2  $\mu$ M, CYP:DOXO (10:1): CYP = 10  $\mu$ M, DOXO = 1  $\mu$ M. Statistical significance (Student's t test) compared to control. ns: not significant; \*\* p<0.01.

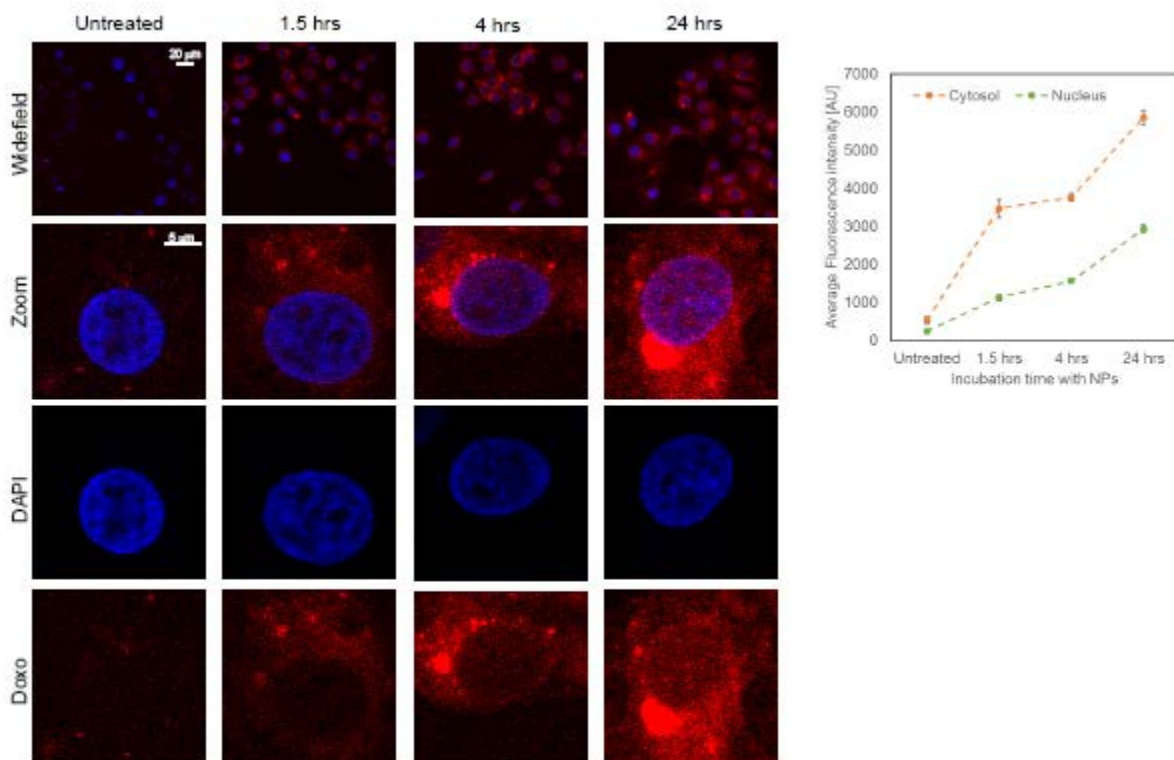


**Supplementary Figure 4.** Cytotoxic activity in IGROV ovarian cancer cell line of cyclophamide, doxorubicin (reported as **Parental drugs**), compounds **1** and **2** not nanoprecipitated (reported as **Conjugates**) and nanoprecipitated compounds **1** and **2** (reported as **NP**). Concentration of the samples: **NP1** (nanoprecipitated CYP-SQ = DOXO-SQ = 10 µM); **NP2** (nanoprecipitated CYP-SQ = 10 µM, DOXO-SQ = 2 µM; **NP3** (nanoprecipitated CYP-SQ = 10 µM, DOXO-SQ = 1 µM; **1:2 (1:1):** CYP-SQ = DOXO-SQ = 10 µM; **1:2 (5:1):** CYP-SQ = 10 µM, DOXO-SQ = 2 µM; **1:2 (10:1):** CYP-SQ = 10 µM, DOXO-SQ = 1 µM; CYP:DOXO (1:1): CYP = DOXO = 10 µM, CYP:DOXO (5:1): CYP = 10 µM, DOXO = 2 µM, CYP:DOXO (10:1): CYP = 10 µM, DOXO = 1 µM. Statistical significance (Student's t test) compared to control. ns: not significant; \*\* p<0.01.

**Comments to Figures 3 and 4.** Supplementary Figure 4 reports the results obtained in IGROV cells, while the effect on the other cell lines are reported in Supplementary Figure 3. Single and combined treatments were performed on exponentially growing cells and are detailed in the Experimental Section. Even the four ovarian cancer cells lines tested showed different degree of sensitivity to the NPs and combined treatment, in all the case NP1 was very effective (NP1 vs NP2, NP2 vs NP3, and NP1 vs NP3, p<0.01). As illustrated in Figure 3, NP1 was the most active NP formulation with a cytotoxic activity similar to CYP:DOXO (1:1) and to DOXO (10µM).

**Confocal microscopy.** We verified the dynamic of the internalization of NP1 into IGROV cells. To this aim, we applied confocal microscopy on cultured cells at different time points (i.e. 1.5, 4 and 24 h) following incubation with hetero-nanoparticles NP1 (Supplementary Figure 5). Longer incubation with the NP1 resulted in the increase of the intracellular fluorescence in the 560-660 nm band, corresponding to the emission of DOXO. Interestingly, for longer incubation times (24 h), DOXO (as free drug or as conjugate compound) is also observed in the cell nucleus. Experimental details. Imaging of IGROV cells was carried out on a Leica SP-5 confocal microscope equipped with a 63x 1.4NA oil-immersion objective. Cells were cultured on #1.5 glass coverslips, incubated with a 1:100 solution of the NP1 and then fixed in 4% PFA, at different times following

addition of NPs. Nuclei were counterstained by incubating the coverslips with 500nM DAPI in PBS for 5 min. Next the coverslips were washed three times in PBS and mounted on microscope slides using Vectashield (VectaLabs). DOXO fluorescence was excited using a 543nm HeNe laser and detected in the 560-660 nm wavelength range. Cytosolic and nuclear fluorescence intensity was carried out automatically by using custom-written routines in matlab. Briefly nuclei are identified by smoothing out the DAPI image with a Gaussian blur filter (size 5 px) followed by segmentation using watershed algorithm. Next the average DOXO intensity in the nucleus was evaluated in by 6-pixels erosion of the regions corresponding to identified nuclei. The average cytosolic intensity was instead calculated on a 6 pixels thick ring surrounding the identified nuclei. All fluorescence intensities were background-subtracted and averaged together. Shown are averages +/- standard errors.



**Supplementary Figure 5.** Confocal microscopy on cultured IGROV cells at different times (i.e. 1.5, 4 and 24 h) following incubation with hetero-nanoparticles **NP1**: CYP-SQ/DOXO-SQ 1:1 (10 μM, 10 μM). Cells were stained with DAPI to visualize nuclei. Nuclear and cytoplasmic average DOXO fluorescence intensities were quantified as described in the Materials and Methods (Shown averages +/- standard errors, n = 15, 32, 102, 81 for 0, 1.5, 4 hrs and 24 h respectively).

### *In vivo* evaluation

**Xenograft experiments.** NOD/SCID/IL2Ry null mice were obtained by Charles River. Two-month old mice were anesthetized with a mixture of 5% xylazine and 10% ketamine in water for injection, and 10,000 A431 cells in 50 µl PBS were injected in the right flank of the mouse. Tumour growth was monitored by palpation twice weekly and, upon tumour appearance, they were measured with a digital caliper. When the tumours reached 3 mm in diameter the chemotherapeutic regimens were administered twice weekly. Ten mice were used per condition for the conditions 1 and 1:2, and 20 mice per condition for control, NP1 and 1. When mice presented signs of distress or weight loss, they were sacrificed. For each condition equal number of male and female mice were used, and littermates were divided equally between the distinct regimens. Mouse colonies were maintained in a certified animal facility in accordance with the European guidelines. All animal experiments were performed in accordance with the guidelines of the relevant Ethical Committee for Animal Welfare (ULB CEBEA) and European guidelines.

**Immunofluorescence.** Frozen sections from cryopreserved tissue, embedded in cryomold (Sakura) using OCT (Tissue Tek), were performed on Superfrost slides (Menzel GmbH) using a cryostat CM-3050-S (Leica) and fixed for 10 min in paraformaldehyde (PFA) 4% in PBS. Non-specific antibody binding was prevented by blocking with 5% Horse Serum (HS), 1% BSA and 0.2% Triton X-100 for 1 h at room temperature (RT). Slides were then incubated overnight at 4°C in the presence of the primary antibodies (anti-Keratin 14, 1:2000 Covence; anti-Vimentin, 1:400 Abcam; Pan-cytokeratin, 1:100 Abcam; Phopsho-Histone-3, 1:600 R&D), followed by 1 h incubation with the secondary antibodies (anti-chicken-Alexa488, anti-mouse-Alexa488, anti-rabbit-RRX, anti-rat-RRX, all 1:400, Jackson ImmunoResearch) at RT. Slides were mounted using Glycergel (Dako) supplemented with 2.5% DABCO (Sigma-Aldrich).