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New doxorubicin nanocarriers based on cyclodextrins

Maurizio Viale[1,*], Valentina Giglio[2], Massimiliano Monticone[1], Irena Maric[1], Giovanni Lentini[3], Mattia Rocco[4], Graziella Vecchio[2,*]

[1]IRCCS A.O.U. San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, U.O.C. Bioterapie, L.go R. Benzi 10, 16132 Genova, Italy;

[2]Dipartimento di Scienze Chimiche, Università di Catania, Viale A. Doria 6, 95125 Catania, Italy;

[3]Dipartimento di Farmacia – Scienze del Farmaco, Università degli Studi di Bari 'Aldo Moro', Via E. Orabona 4, 70126 Bari, Italy;

[4]IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, U.O.S. Biopolimeri e Proteomica, IRCCS A.O.U. San Martino-IST, L.go R. Benzi 10, 16132, Genova, Italy.

Corresponding author: Fax. +390105558373, email: maurizio.viale@hsanmartino.it.

Dedicated to the memory of Dr. Carmela Spatafora

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Summary

Polymeric nanoparticles and fibrin gels (FBGs) are attractive biomaterials for local delivery of a variety of biotherapeutic agents, from drugs to proteins. We combined these different drug delivery approaches by preparing nanoparticle-loaded FBGs characterized by their intrinsic features of drug delivery rate and antiproliferative/apoptotic activities. Inclusion complexes of doxorubicin (DOXO) with oligomeric β-cyclodextrins (oCyD) functionalized with different functional groups were studied. These nanocarriers were able to interact with FBGs as shown by a decreased release rate of DOXO. One of these complexes, oCyDNH_2/DOXO, demonstrated good antiproliferative and apoptotic activity *in vitro*, reflecting a higher drug uptake by cells. As hypothesized, the nanocarrier/FBG complexes showed a lower drug release rate than similar FBGs loaded with the corresponding non-functionalized oCyD/DOXO.

Taken together, our results provide experimental evidence that oCyDNH_2/DOXO complexes may be useful components in enhanced FBGs and further build support for the great promise these complex molecules hold for clinical use in localized anticancer therapy of inoperable or surgically removable tumors of different histological origin.

Keywords: Doxorubicin; Cyclodextrin nanocarrier; Tumor targeting; Fibrin gels; Localized treatment.

Introduction

Biodegradable and biocompatible polymeric nanoparticles hold great promise as drug delivery systems because of their high stability, drug loading capacity, and controllable drug-release profiles. Furthermore, by chemical modification of their surfaces polymeric nanoparticles can be modified to optimise their use for specific purposes, such as agents for drug delivery.

Cyclodextrin (CyD)-based nanocarriers, beside their ability to encapsulate guest molecules, can also improve drug stability and efficiently regulate its release rate [1-6]. CyD polymers exhibit a good enhancement of the inclusion ability towards various guests [7,8]. A successful example of linear CyD polymers designed specifically to overcome some limitations in the systemic transport of a drug is Cyclosert Platform [9]. CRLX-101 is a nanoparticle based on the Cyclosert delivery platform that consists of the drug camptothecin conjugated to CyD polymers and is currently in clinical trials for cancer nanotherapy [10]. CALAA-01 is a nanoformulation carrying siRNA for targeted RNA interference (RNAi) therapy [11]. These nanoparticles are prepared via the self-assembly of CyD-containing polymers in the presence of nucleic acids. CALAA-01 is currently undergoing a Phase II clinical trial against melanoma; it has demonstrated evidence of successful RNAi in humans [12]. CyD-based nanoparticles have been used as nanocarriers of LA-12, a prodrug of cisplatin [13], and the results suggested that CyD polymers could be very promising nanocarriers for poorly water-soluble anticancer drugs. To increase their drug loading features, CyD polymers have also been modified with anionic or cationic groups [14]. More recently, the synthesis of oligomers has also been exploited, since drug carriers with low molecular weight have some advantages such as an easy excretion without degradation by renal tubules [15].

Doxorubicin (DOXO) is one of the most powerful anthracycline anticancer drugs, and is used in the treatment of leukemia and various solid tumors despite of drawbacks associated with poor water solubility, severe cardiotoxicity, and emerging multidrug resistance [16]. Methyl CyDs have been investigated as potential carriers for DOXO, due to their ability to improve its activity [17] and to promote its release in brain tissues [18]. CyD nano-assemblies have also shown great potential to improve the pharmacological profile of DOXO [19]. A strategy to approach these issues has been the development of suitable carriers optimizing the drug administration, such as PEGylated liposomal Caelyx/Doxil and not PEGylated liposomal Myocet [20].

We report here a comprehensive study of inclusion complexes of DOXO with oligomeric β-CyDs (oCyD) with different functional groups, such as amino groups ($oCyDNH_2$) and the folate residue (oCyDFA). The folic acid (FA) moiety could permit the recognition of folate receptors (FR) identified as an attractive tumor marker overexpressed on the surfaces of several cancer cells [21]. Instead, $oCyDNH_2$ was selected for the positive charge at

physiological pH [22] that could increase the uptake of the drug or the interaction with the negatively charged fibrin gels (FBGs) [23] used as loco-regional carriers.

FBGs are a well-studied vehicle for the delivery of various therapeutic biomolecules, including the chemotherapeutics cisplatin, DOXO [24,25], 5-fluoruracil, mitomycin C, and enocitabine [26]. The release kinetics from FBGs can be modified through affinity, composition and covalent linkage. FBGs are formed by polymerization of fibrinogen (FG) following activation by the enzyme thrombin in a solution containing calcium ions. FBGs have physical and chemical properties that make them widely adaptable and highly biocompatible, and, as such, may represent one of the best options for localized drug delivery after surgical removal of tumors. Furthermore, some studies illustrate the possibility of fibrin gels as a carrier for other drug delivery systems such as nanoparticles and composite gels [25, 27].

In a previous study, by linking together the drug release control exerted by our complexes and nanoparticles with the already characterized FBGs drug delivery system, we showed that tumor exposure and Pt tumor concentration may last for several days [27]. This allowed the long lasting exposure to cisplatin and, in particular, cisplatin-hyaluronate complexes (DDP-HA).

In the present work, we used CyD oligomers (oCyD, oCyDNH_2 and oCyDFA) as nanocarriers of DOXO and validated their cytotoxicity and antiproliferative activity in both DOXO-sensitive and DOXO-resistant cell lines. In addition, in order to design effective drug release systems based on FBGs, we evaluated their ability to bind FG.

Materials and methods

Chemicals

All chemicals obtained from commercial sources were used without further purification. CyD oligomer of about 8 CyD cavities (oCyD, 12 kDa, 65% CyD), amino-oligomer (oCyDNH_2, 12 kDa, 70% CyD) and its conjugate with folic acid (oCyDFA) were synthesized as described elsewhere [13, 22]. Briefly, oCyD was synthesized starting from β-CyD. The polymerization reaction was carried out under basic conditions using epichlorohydrin as the cross-linking agent [3]. Under similar experimental condition oCyDNH_2 was synthesized starting from (tert-butyloxycarbonyl)-protected 3A-amino-3A-dideoxy-2A(S), 3A(R)-β-cyclodextrin [2]. Zeta potential of oCyDNH_2 is 7.1 ± 1 mV at pH = 7.4.

Inclusion complexes of DOXO and oCyDs were prepared by mixing the stock solution of DOXO with aqueous solutions of CyD oligomers. Host/DOXO 1:5 molar ratios were tested.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell proliferation assays were performed on cancer cell lines differing in their degree of folic acid receptor expression [21]. All inclusion complexes with DOXO were assayed in all sensitive and DOXO-resistant cell lines.

The following human cancer cell lines were used: SK-Mel-28 (melanoma), IMR-32 (neuroblastoma), SHSY5Y (neuroblastoma), LAN-5 (neuroblastoma), A2780 (ovarian carcinoma), Hep-G2 (hepatoma), MDA-MB-231 (breast), MCF-7 (breast), A2780/DX3 (ovarian carcinoma with overexpression of MDR [28]). Cells were plated at the appropriate concentration and opportune medium into flat-bottomed 96-well microtiter plates. After 6–8 h incubation, they were treated with 20 μL of different concentration of DOXO (reference drug), oCyD/DOXO, oCyDNH$_2$/DOXO, and oCyDFA/DOXO, in a dilution ratio of 1:5, in order to reach a final starting concentration on cells of 1 μM (except for A2780/DX3 where it was 10 μM). Cell cultures were then processed by the MTT assay after 3 days, as described elsewhere [29]. The stock solutions of all preparations were prepared in normal saline and aliquoted at -20 °C.

IC_{50} values were calculated from the analysis of single concentration–response curves, each final value being the mean of 4–11 experiments.

Visualization of apoptotic cells/nuclei by 4-6-diamidino-2-phenylindole (DAPI) staining

A2780, IMR-32 and MDA-MB-231 cells were plated into 24-well microtiter plates in 1.4 mL at 1.0, 1.2 and 0.8×10^4 cells/well, respectively. After 6-8 h, all compounds were added to a final concentration of 100 nM or 200 nM in the case of MDA-MB-231 cells. After 72 h, cells were harvested, washed three times with PBS and fixed with 100 μL of 70% ethanol in PBS. Samples were then maintained at 4 °C until examined microscopically for percentage of apoptotic segmented nuclei/cells, after first adding 5 μL of a 10 μg/mL DAPI solution in water.

Cytofluorimetric study of intracellular accumulation of DOXO and cyclodextrins complexes

A2780 and IMR-32 cells were plated in 24-well plates in 0.8 mL at 1.0 and 1.2×10^5 cells/well, respectively. After incubation at 37 °C for 16 hours and cells had reached 75-85% confluence, they were treated with 2 μM oCyD/DOXO or oCyDNH$_2$/DOXO. After 1 hour cells were detached by exposure to trypsin-EDTA at 37 °C for 5 min, washed twice with cold PBS, counted, and fixed with 0.5 mL of 3.7% para-formaldehyde in PBS containing 2% sucrose. Untreated cells were assayed as well. The intracellular mean fluorescence intensity (MFI) of cells was determined by flow cytometry (CyAn ADP cytometer, Beckman Coulter, Roma, Italy) using 493 nm excitation and 636 nm emission bandpass filters. Values were normalized in arbitrary units calculated as: [MFI of treated cells - MFI of control cells]/MFI of control cells.

In vitro DOXO release kinetics

The kinetics of DOXO release from oCyD/DOXO and oCDNH_2/DOXO inclusion complexes was studied *in vitro* in 48-well plates at 37 °C. Aliquots (400 μL) of FBGs (22 or 40 mg/mL) loaded with equal amount of DOXO (18.6 μg) included in oCyD/DOXO and oCyDNH_2/DOXO complexes were seeded and, after gel formation at 37 °C for 30 min in the presence of 0.81 mM Ca^{2+} and 1.33 U thrombin/mg FB, were overlaid with 900 μL normal saline. After incubation at 37 °C (t =0, 5, 15, 30, 60, 120 and 360 min, and 24-72 or 24-196 h), the supernatants were removed and analyzed for DOXO content by measuring the absorbance at 488 nm using an extinction coefficient of 11,500 $L \cdot mol^{-1} \cdot cm^{-1}$ [30].

Statistical analysis

Non-parametric data were analyzed by the Mann-Whitney test.

Results and discussion

Antiproliferative activity (MTT assay)

The results of the antiproliferative assay are shown in Table 1. To begin with, our assays indicate that the CyD polymers/DOXO complexes mainly have an antiproliferative activity on average not substantially different to that of DOXO alone [median (min-max) IC_{50}s: 73.0 μM (5.6-277.7 μM), 105.4 μM (7.3-535.8 μM), 117.8 μM (7.0-449.3 μM), 82.8 μM (4.9-435.0 μM), for DOXO, oCyD/DOXO, oCyDFA/DOXO, and oCyDNH_2/DOXO, respectively] (Table 1). However, although oCyDNH_2/DOXO in general displays an activity similar to the parent compound DOXO, in once instance, IMR-32 cells, it is notable that a significant reduction of the IC_{50} (108 ± 5.5 μM vs 61.1 ± 10.9 μM, $p = 0.029$) is observed.

The most significant result is a better activity of oCyDNH_2/DOXO compared to oCyD/DOXO. This is particularly evident for cell lines IMR-32 ($p = 0.016$), LAN-5 ($p = 0.029$), A2780 ($p = 0.002$) and MDA-MB-231 ($p = 0.038$), with a mean IC_{50} reduction in these cells of 41.0 ± 7.4%.

oCyDFA/DOXO, when compared to oCyD/DOXO did not show any IC_{50} reduction demonstrating that addition of a folate group had no significant impact on antiproliferative activity. This was true even in A2780 cells over-expressing the folic acid receptor. This is probably due to the low amount of functionalization (the extent of modification with FA was only 15% of the cavities) and/or the low accessibility of FA by the receptor [13]. Moreover, in no cases oCyDFA/DOXO displays an activity higher than that of DOXO alone (Table 1).

Regarding the treatment of MDR1 resistant A2780/DX3 cells with DOXO and CyD polymers/DOXO, only oCyDNH_2/DOXO shows a trend towards an antiproliferative activity greater than that of DOXO alone (-23%,

$p < 0.10$, Table 1). In any case, this antiproliferative activity was much lower than that observed for DOXO on the sensitive counterpart A2780 cells, revealing the absence of a possible overcoming of drug resistance (Table 1).

Apoptosis triggering

Our data in Fig. 1 show that, at identical concentrations of the anticancer drug, in IMR-32 cells oCyDNH$_2$/DOXO has an activity significantly higher than DOXO ($p = 0.036$), while versus oCyD/DOXO and oCyDFA/DOXO only a trend is apparent ($p = 0.069$). It is noteworthy that these results are superimposable to those obtained by the MTT assay for the evaluation of antiproliferative activity.

In MDA-MB-231 cells, while oCyDNH$_2$/DOXO triggers apoptosis similarly to DOXO (Fig. 1), both oCyD/DOXO and oCyDFA/DOXO induce apoptosis to a lesser extent than oCyDNH$_2$/DOXO and DOXO (oCyDNH$_2$/DOXO vs oCyD/DOXO and oCyDFA/DOXO, $p = 0.027$ and $p = 0.005$, respectively; DOXO vs oCyD/DOXO and oCyDFA/DOXO, $p < 0.003$ and < 0.001). In this case as well we found a good correspondence between the apoptosis triggering and the antiproliferative activity (Fig. 2).

Finally, in A2780 cells we did not find a correlation between the antiproliferative activity and the triggering of apoptosis or significant differences between treatments, as found in IMR-32 and MDA-MB-231 cells.

DOXO uptake

In order to establish whether the difference in antiproliferative activity was caused by a differential exposure of target cells to DOXO, we evaluated by cytofluorimetry the drug uptake by A2780 and IMR-32 cells after administration of the same concentrations of oCyD/DOXO or oCyDNH$_2$/DOXO. Our data show that the intracellular accumulation of DOXO is dependent on the form of the cyclodextrin-based carrier utilized for drug delivery. The drug uptake of oCyDNH$_2$/DOXO was significantly higher or showed a trend compared to that of oCyD/DOXO in all cell lines tested (A2780: 5.99 ± 0.68 MFI vs 6.88 ± 0.44 MFI, $n = 5$-6, $p = 0.009$; IMR-32: 3.83 ± 0.93 MFI vs 5.15 ± 1.09 MFI, $n = 4$, $p = 0.056$). It is noteworthy that also in this case the results of DOXO uptake correlates with the results of antiproliferative activity for these two CyD polymers utilized, thus demonstrating that the antiproliferative activity of our complexes correlate with the ability of the cyclodextrin nanocarrier to target the cells and release the anticancer drug (Fig. 2).

In vitro release kinetics

The results of *in vitro* release kinetics of DOXO from FBGs loaded with oCyD/DOXO or oCyDNH$_2$/DOXO under total recovery conditions reveal that, at the constant concentration of thrombin and Ca^{2+} per mg FG and at the

concentration of 22 mg/mL FG, the release of the anticancer drug is lower for oCyDNH$_2$ (Table 2). It is of note that with FBGs at the higher FG concentration (40 mg/mL) we did not observe significant differences of drug release between oCyD/DOXO and oCyDNH$_2$/DOXO. This last result emphasizes the importance of FG concentration in FBGs for the control of drug release in our systems.

Conclusions

In this work we have reported the use of cyclodextrin-based nanoparticles as DOXO carriers. Neutral β-cyclodextrin oligomers and derivatives with amino-groups or FA were used. All these systems are able to include DOXO. Inclusion complexes of DOXO with the amino oligomer oCyDNH$_2$ show a higher activity than DOXO alone in IMR-32 cells, and a slight increased activity in the A2780/DX3 DOXO-resistant cells. Contrary to expectations, no improvement of antiproliferative activity was found by functionalization with FA, as previously reported by us in the case of LA-12 [12]. This result is likely attributable to the low degree of functionalization and/or the low accessibility of FA by the receptor.

Another important goal of our work was to prepare DOXO-cyclodextrin complexes able to interact with the physico-chemical features of FBGs, resulting in a more controlled release of DOXO. We hypothesized that oCyDNH$_2$, which is positively charged at physiological pH, could significantly interact with FG and likely with the fibrin network in FBGs, which are mainly negatively charged at neutral pH. This hypothesis was confirmed by preliminary experiment of *in vitro* release kinetics demonstrating longer rates of release for DOXO when it was loaded on the cationic oligomeric β-cyclodextrin oCyDNH$_2$.

Taken together, our results suggest that oCyDNH$_2$ could favour drug loading and a possible long-lasting and controllable release of anticancer drugs *in vivo*. It remains to evaluate the effect of different concentrations of thrombin and Ca^{2+} ions on the release kinetics of this DOXO-cyclodextrin complex, and the importance of these factors in *in vivo* experiments.

Finally, our results open the possibility of exploiting the oCyDNH$_2$/DOXO complex for its use as a component of new FBGs. These composite FBGs could be used for the loco-regional treatment of inoperable tumors or for the “sterilization” of the tumor site after its removal, preventing recurrences and the spread of metastatic cells.

Figure 1

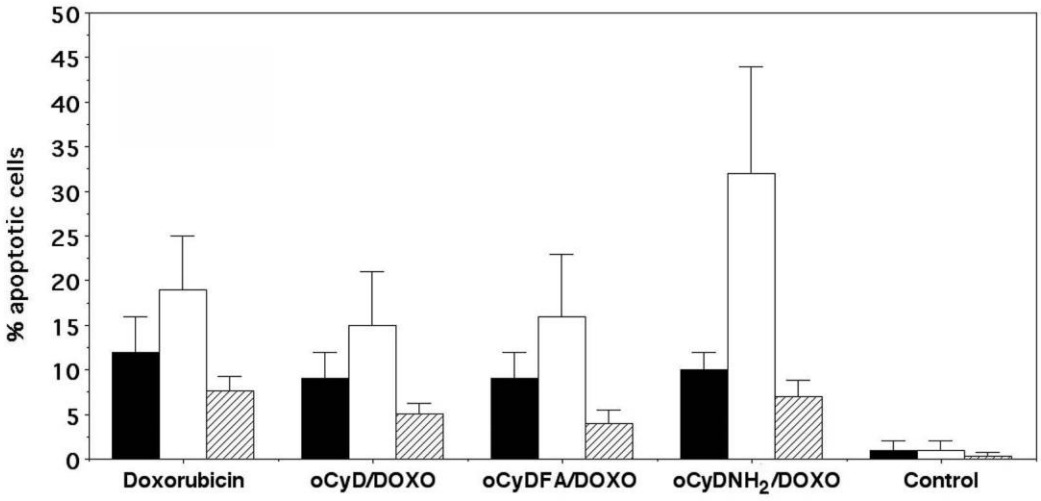

Fig. 1 Apoptosis triggered by DOXO, oCyD/DOXO, oCyDNH$_2$/DOXO, and oCyDFA/DOXO in A2780, IMR-32 and MDA-MB-231 cells. Bars represent the mean ± SD of apoptotic cells. A2780 (■), n=7-11; IMR-32 (□), n=7-8; MDA-MB-231 (▨), n=7-8). See text for p values.

Figure 2

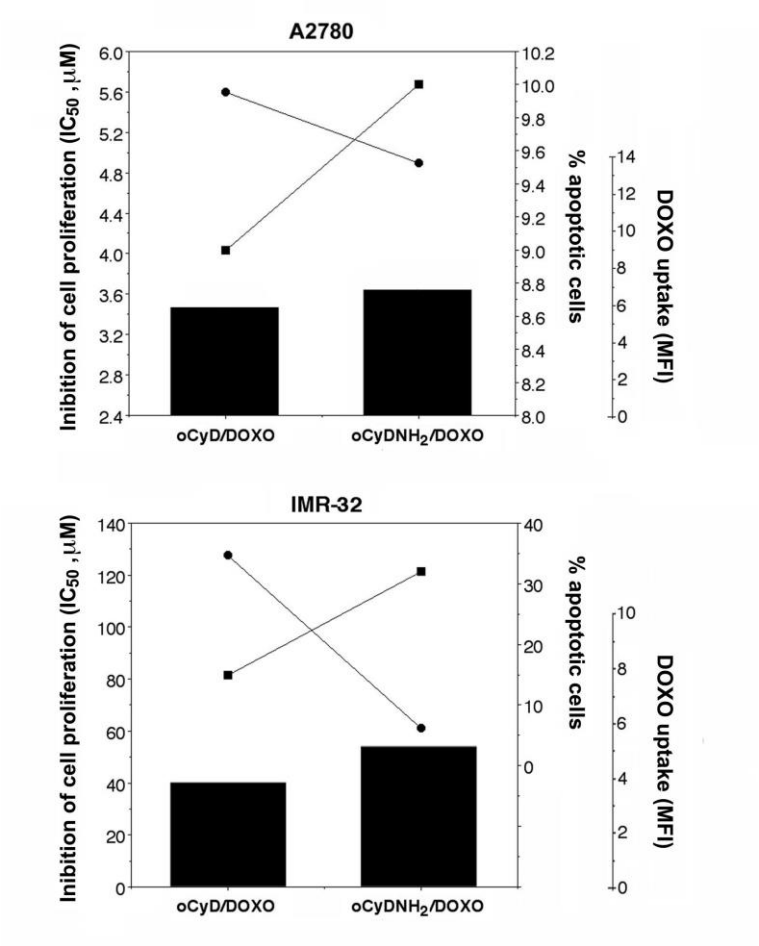

Fig. 2 Antiproliferative activity, apoptosis triggering, and doxorubicin uptake in A2780 and IMR-32 cells. The figure represents the relationship between the mean IC_{50} (●), the percent of apoptotic cells after treatment with 100 nM DOXO (■), and the DOXO uptake after the exposure to 2 μM concentration (bars) of A2780 and IMR-32 cells treated with oCyD/DOXO and oCyDNH$_2$/DOXO. The variance of means is reported in the text, in Table 1, and Fig. 1.

Table 1. Antiproliferation activity of doxorubicin and doxorubicin complexes in human tumor cell lines.

Cell line	IC_{50} (nM)[a]			
	DOXO	oCyD/DOXO	oCyDFA/DOXO	oCyDNH$_2$/DOXO
SKMel28	77.7±6.9	94.0±35.7	73.2±12.4	97.7±13.5
IMR-32	108.0±5.5	127.6±24.8	127.7±6.6	61.1±10.9
SHSY5Y	90.3±7.8	105.4±36.1	117.8±30.7	76.7±5.3
LAN-5	73.0±15.2	94.8±13.1	87.6±12.9	60.8±17.1
A2780	5.6±1.3	7.3±2.7	7.0±2.6	4.9±1.6
Hep-G2	126.7±17.6	211.6±64.3	248.4±88.6	209.5±70.9
MDA-MB-231	65.7±16.4	145.9±59.1	130.0±39.0	82.8±30.0
T47D	277.7±101.3	535.8.±147.1	449.3±154.4	435.0±71.4
MCF-7	62.0±15.1	101.1±38.4	107.2±29.3	96.5±30.2
A2780/DX3	1.39±0.28	1.58±0.29	1.75±0.26	1.07±0.30

[a]IC_{50} values reported as mean ± SD (n = 4-17)

Table 2. Kinetics of DOXO release *in vitro* from FBGs loaded with oCyD/DOXO or oCyDNH$_2$/DOXO

Complex	22 mg/ml FBGs		40 mg/ml FBGs	
	$t_{50\%}$	$t_{80\%}$	$t_{50\%}$	$t_{80\%}$
oCyD/DOXO	11.2±1.6[a,b]	44.6±11.0[c]	31.2±3.7	92.6±16.1
oCyDNH$_2$/DOXO	14.2±3.2	56.9±5.3	29.4±2.6	90.0±15.6

[a] $t_{50\%}$ and $t_{80\%}$ are expressed in hours and obtained from mean curves derived from 4-7 experiments.

[b] $p = 0.056$ vs oCyDNH$_2$/DOXO.

[c] $p = 0.048$ vs oCyDNH$_2$/DOXO.

Conflict of interest

Maurizio Viale declares that he has no conflict of interest. Valentina Giglio declares that she has no conflict of interest. Massimiliano Monticone declares that he has no conflict of interest. Irena Maric declares that she has no conflict of interest. Giovanni Lentini declares that he has no conflict of interest. Mattia Rocco declares that he has no conflict of interest. Graziella Vecchio declares that she has no conflict of interest.

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Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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