

Rapid communication

# Liberation of doxorubicin from HPMA copolymer conjugate is essential for the induction of cell cycle arrest and nuclear fragmentation in ovarian carcinoma cells

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## Abstract

Despite intensive study, the molecular mechanism for cell toxicity of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-bound doxorubicin remains unclear. Moreover, the ability of the released drug to accumulate in the nucleus has also been questioned. We have hypothesized that the pattern of cell cycle progression is a useful indicator for the presence of free doxorubicin in the nucleus and its interaction with nuclear DNA. The effects of HPMA copolymer-bound doxorubicin on cell cycle progression were evaluated in this study in cultured human ovarian cancer A2780 cells. We determined that P-GFLG-DOX, but not P-GG-DOX, initiates cell cycle arrest and nuclear fragmentation in the same manner as free DOX, but with a time-delay. Our data indicate that drug release from the conjugate is required for the apoptotic activity associated with the conjugate.

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**Keywords:** HPMA copolymer; Doxorubicin; Cell cycle; Ovarian cancer

## 1. Introduction

Natural and synthetic polymers have been widely used as anticancer drug carriers for several decades [1,2]. Conjugation of drugs to the macromolecular carrier increases their circulating time in the bloodstream, decreases systemic toxicity, allows its passive accumulation in tumor tissue due to enhanced permeability and retention (EPR) effect [3] and intracellular uptake by endocytosis (reviewed in [4]). The incorporation of peptide linkers, which are degradable in the lysosomal compartment, is one of the critical aspects for the design of macromolecular therapeutics. Detailed studies performed previously in our group have provided the theoretical basis and

experimental evidence for drug release following enzymatic degradation in lysosomes of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-drug conjugates containing biodegradable tetrapeptide linker (P-GFLG-DOX) [4,5].

Designed for lysosomotropic drug delivery, P-GFLG-DOX showed pronounced cytotoxic activity in various experimental models. It has been shown that P-GFLG-DOX overcame drug efflux pumps and ceased cancer cell proliferation by inhibiting DNA repair, replication, and biosynthesis, and thereby inducing apoptosis [6–9]. Furthermore, P-GFLG-DOX was shown to be efficacious in the treatment of drug-resistant tumors [10], and has been tested in several clinical trials with encouraging results [11]. However, the molecular mechanism of toxicity at the cellular and subcellular levels has remained unclear. Recent studies have raised concerns about the role that endocytic internalization pathways and lysosomal degradation of the polypeptide linker play to dictate the intracellular fate of the conjugate. Based on the results of fluorescent microscopy, it has been reported that no free doxorubicin was detected in the

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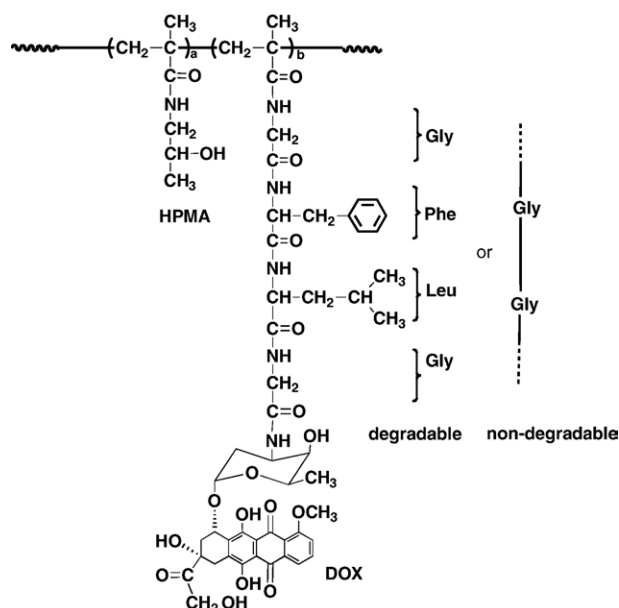


Fig. 1. Structure of HPMA copolymer-DOX conjugates. P-GFLG-DOX conjugate contained 0.11 mmol doxorubicin/g polymer (6.4 wt.-%); 0.08% of free DOX (of total drug content); molecular weight, Mw=27 kDa. P-GG-DOX conjugate contained 0.18 mmol doxorubicin/g polymer (9.5 wt.-%); 0.04% of free DOX (of total drug content); molecular weight, Mw=25 kDa.

nuclei of cells treated with P-GFLG-DOX. It was concluded that the conjugate educed its cellular toxicity mainly through its effects on cellular membranes, and necrosis was suggested as the major mode of cytotoxicity [12,13]. On the other hand, other reports provided evidence for the accumulation of doxorubicin released from HPMA copolymer conjugates in the nuclei [14–16]. Both necrotic and apoptotic types of cell death were reported [7–9,15–18].

To test the accessibility of liberated DOX to the nuclei, we took advantage of the fact that DOX could be attached to the copolymer through degradable or non-degradable oligopeptides (Fig. 1). Both conjugates, P-GFLG-DOX and P-GG-DOX, were extensively purified from residual free DOX by chromatography. The ability of the two types of conjugate to affect cell proliferation and cell cycle progression in cultured ovarian cancer cells was evaluated.

## 2. Materials and methods

### 2.1. Chemicals

DOX was a kind gift from Dr. A. Suarato, Pfizer, Milano, Italy. HPMA copolymer-DOX conjugates, P-GFLG-DOX and P-GG-DOX, were synthesized by a polymer analogous reaction of DOX with an HPMA copolymer precursor synthesized as previously described [19]. Both conjugates were extensively purified from residual free drug by chromatography (for details see supplemental Fig. S2). Stock solutions of each drug were prepared in distilled water, filtered through a 0.2  $\mu\text{m}$  membrane,

aliquoted, and stored at  $-20\text{ }^{\circ}\text{C}$ . Fetal bovine serum (FBS) and media supplements were purchased from HyClone Laboratories (Ogden, UT); cultured media and insulin were purchased from Sigma-Aldrich (Milwaukee, WI).

### 2.2. Cells

The A2780 cell line, a human ovarian carcinoma was obtained from T.C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were grown in RPMI 1640 media supplemented with 10% FBS, 2 mM glutamine, and 10  $\mu\text{g}/\text{ml}$  insulin at  $37\text{ }^{\circ}\text{C}$  in 5%  $\text{CO}_2$  and 95% humidified air. The cells were maintained in logarithmic phase of growth throughout of all experiments. Cell growth inhibition was measured by modified MTT (Dojindo Molecular Technologies, Gaithersburg, MA) colorimetry as described previously [8]. Cell cycle progression was monitored by the flow cytometric measurement of DNA content. Analysis of DNA content in cells stained with propidium iodide was performed using FACScan (Becton Dickinson, Mountain View, CA). The percentage of cells in each phase of the cell cycle was evaluated using the ModFit software (Verity Software House, Topsham, ME). Nucleus fragmentation and apoptotic body formation were visualized in fixed cells stained with DAPI by a laser scanning confocal microscope Olympus FluoView<sup>®</sup> FV1000 (Olympus America Corp., Center Valley, PA). The objective specifications were 60x oil immersion and numerical aperture 1.42.

### 2.3. Statistical Analysis

Experimental data were analyzed using single or two-factor analysis of variance (ANOVA) where appropriate. The difference between values was considered significant if  $p < 0.05$ .

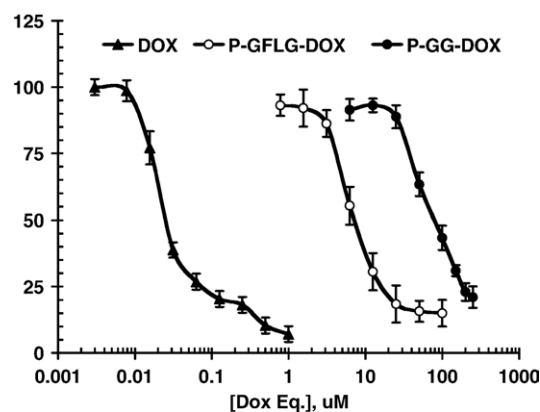


Fig. 2. Cytotoxicity of three different forms of DOX to ovarian carcinoma A2780 cells. Cells were incubated with each drug for 72 h. Amounts of cells were evaluated using modified MTT assay (Dojindo Molecular Technologies). 1 – DOX; 2 – P-GFLG-DOX; 3 – P-GG-DOX. IC<sub>50</sub> values: DOX- 0.05 ± 0.01  $\mu\text{M}$ ; P-GFLG-DOX- 7.4 ± 0.8  $\mu\text{M}$ ; P-GG-DOX- 83.0 ± 4.7  $\mu\text{M}$ .  $p < 0.001$ . The data were analyzed using single factor analysis of variance (ANOVA). Mean values ± S.D. from 3 or 4 independent experiments are shown.

### 3. Results and discussion

Notably, all three forms of DOX, even P-GG-DOX, inhibited proliferation of A2780 cells (Fig. 2). The degradability of P-GFLG-DOX in the presence of rat liver tritosomes (i.e., a mixture of lysosomal enzymes) was confirmed (supplemental Fig. S1 A). This conjugate showed almost 11-fold higher toxicity toward A2780 cells compared to P-GG-DOX. No degradation of P-GG-DOX by tritosomes was detected in recent experiments (supplemental Fig. S1 B). Observed toxicity of P-GG-DOX was expected. Based on published data, DOX cell surface-directed interaction and toxic influence on non-nuclear intracellular systems are suspected [20,21]. Apparently, the detailed explanation of the mechanism of P-GG-DOX toxicity demands further investigations in this direction.

Cell cycle analysis demonstrated that both free DOX and P-GFLG-DOX arrested A2780 cells in the G2 phase of the cell cycle in a concentration ( $p < 0.001$  for both drugs) and time-dependent ( $p < 0.001$  for both drugs) manner (Figs. 3 and 4). However, in cells treated with P-GFLG-DOX, the appearance of populations shown to be in G2 was delayed at least for 4–6 h compared to those treated with free DOX. Further, the

accumulation of cells in G2 was accompanied by a 6–8 fold decrease in the number of cells in S phase compared to control cells, an indication of DNA replication inhibition. P-GG-DOX had no effect on cell cycle progression when applied using the same range of concentrations as P-GFLG-DOX. P-GG-DOX induced a slight increase in the number of cells in G2 when used at equitoxic ( $2 \times IC_{50}$ ) concentrations as P-GFLG-DOX. However, no statistically significant concentration- ( $p > 0.5$ ) or time-dependence ( $p > 0.5$ ) was observed in case of P-GG-DOX (Fig. 4). Contrarily to P-GFLG-DOX, about two times higher portion of cells treated with P-GG-DOX remained in S phase, indicating lesser inhibition of DNA replication.

Cell cycle arrest can trigger specific cellular responses, resulting in apoptotic cell death. To test whether drugs induce apoptosis, A2780 cells were incubated with  $2 \times IC_{50}$  concentrations of either free DOX or the conjugates for different periods of time, and then stained with nuclear dye. Nuclear fragmentation, one of the morphological signs of apoptosis [22], was observed in A2780 cells after 14 h incubation with free DOX, and after 24 h incubation with P-GFLG-DOX (Fig. 5). No signs of nuclear fragmentation were found in cells treated with P-GG-DOX after 24 h, but it appeared that most cells possessed

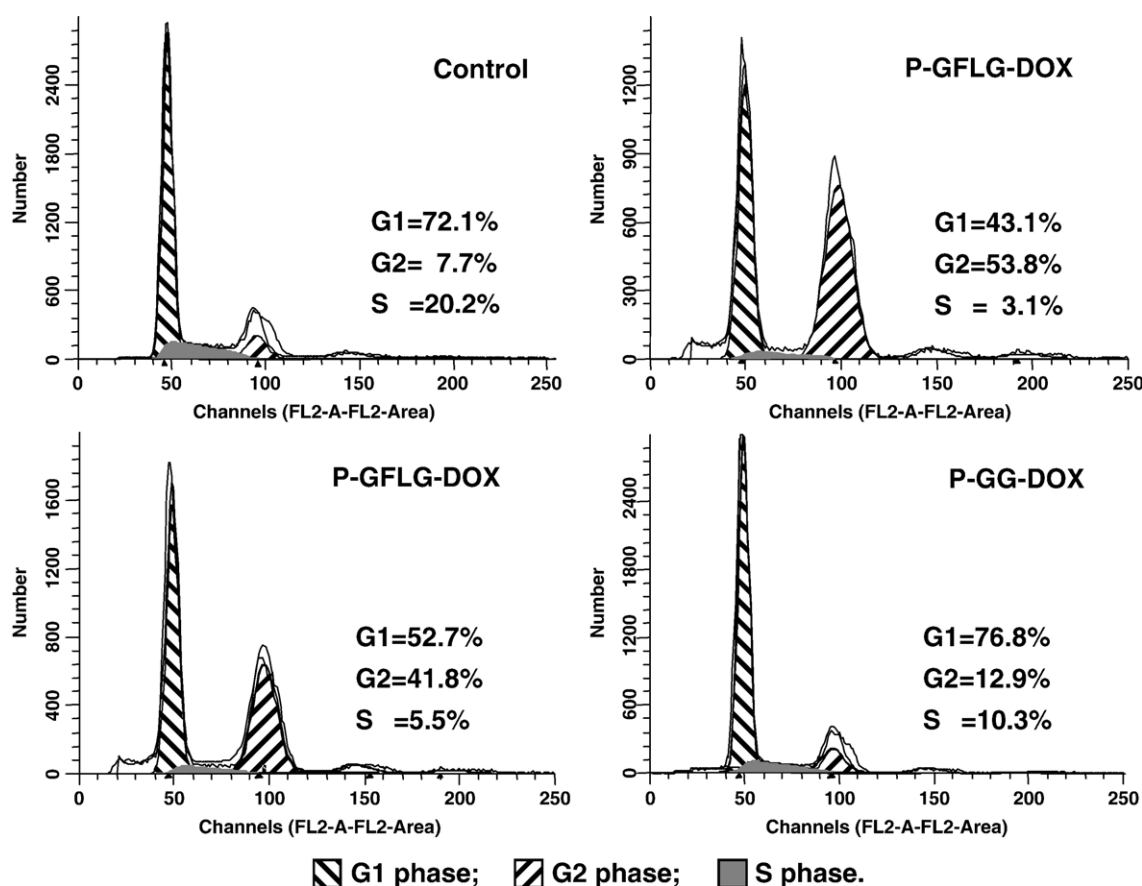


Fig. 3. Changes of DNA content in A2780 cells after treatment with different forms of DOX. Cells were incubated with  $2 \times IC_{50}$  concentrations of different forms of DOX for 24 h, harvested, stained with propidium iodide, and analyzed on FACScan (Becton Dickinson). The percentage of cells in each phase of the cell cycle was evaluated using the ModFit software (Verity Software House). Representative histograms from one experiment are shown.

enlarged, swollen nuclei. Cells treated with P-GG-DOX appeared to be more homogeneous than cells treated with free DOX or P-GFLG-DOX. In the latter two cases, cells with condensed, swollen, or fragmented nuclei could be observed even within a single field of view. Cells undergoing mitosis were never found among cells treated with free DOX or P-GFLG-DOX. However, mitotic cells were occasionally seen among the cells treated with P-GG-DOX. These findings indicate a high diversity of cellular responses for a given drug treatment and this variability should always be considered when characterizing the mode of cell death [23].

DOX can cause cell cycle arrest by initiation of DNA damage through several proposed mechanisms that require the presence of drug in the nucleus and direct interaction with genomic DNA [21]. Our data suggest that HPMA copolymer-bound DOX initiates the same events in the nucleus as free DOX, but with a time-delay. Observed differences in time-dependence and time-delay for both conjugates ruled out involvement of residual free DOX in the nuclear events. On the

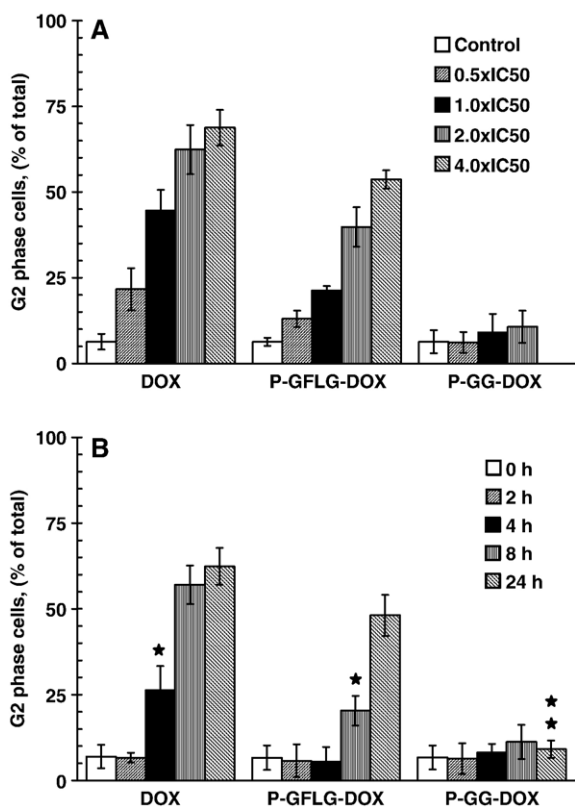


Fig. 4. Accumulation of A2780 cells in the G2 phase of the cell cycle after drug treatment. Cells were prepared and data acquired and analyzed as described in the legend to Fig. 3. Mean values  $\pm$  S.D. from 3 independent experiments are shown. The data were analyzed using two-factor analysis of variance (ANOVA). A- Concentration dependence. Cells were incubated with each drug for 24 h. The abscissa represents the concentration of DOX expressed as the fraction of IC<sub>50</sub> measured after 72 h of incubation. B- Kinetic accumulation of A2780 cells in G2 phase of cell cycle. Cells were incubated with 2xIC<sub>50</sub> concentration of each drug. \*  $p < 0.001$ . Statistical comparisons were made between control and treated cells. \*\*  $p < 0.001$ . Statistical comparisons were made between P-GFLG-DOX and P-GG-DOX treated cells only at 24 h.

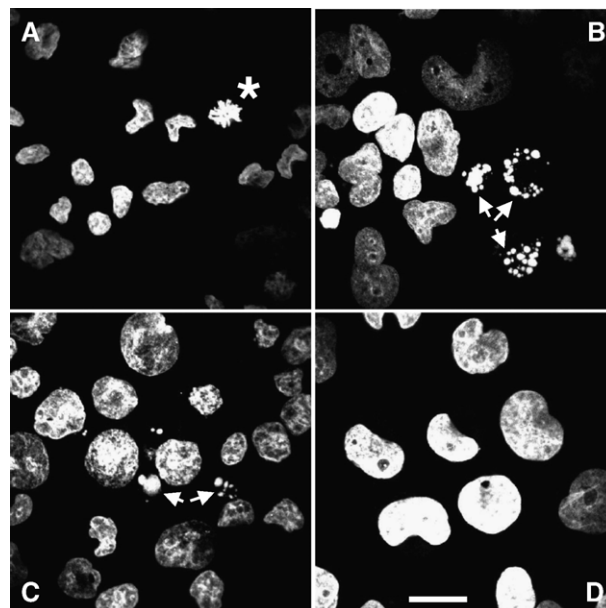


Fig. 5. Nuclear fragmentation and apoptotic body formation in A2780 cells treated with drugs. A2780 cells were incubated with  $2 \times$  IC<sub>50</sub> concentrations of each drug, fixed, and stained for DNA with DAPI. Cells were visualized using a confocal fluorescent microscope Olympus FV1000. Asterisk indicates cell undergoing mitosis. Arrows indicate cells with fragmented nucleus. Bar size -20  $\mu$ m. A- control; B - DOX, 14 h incubation; C- P-GFLG-DOX, 24 h incubation; D- P-GG-DOX, 24 h incubation.

contrary, the data showed that DOX liberated from the conjugate was the agent responsible for cell cycle arrest.

These data are in good agreement with our previous studies, which revealed that the P-GFLG-DOX initiates a time-delayed expression of proteins and apoptosis induction that was also elicited by free doxorubicin [8,9]. Interestingly, time-delays for the activity of P-GFLG-DOX was also reported previously by another group, however, it was interpreted as due to differences in the mechanism of action of the conjugate compared to free DOX or HPMA copolymer-bound DOX containing hydrazone bonds [24–26].

#### 4. Conclusion

Our study demonstrated that P-GFLG-DOX can inhibit proliferation and induce apoptosis of cancer cells, as long as the experimental conditions permit endocytosis of the macromolecular therapeutics and lysosomal degradation of the conjugate. The degradability of the oligopeptide side-chains determined whether or not doxorubicin accumulated in the nucleus, as well as the resulting kinetics of cell cycle regulation, and possibly, the induction of apoptosis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2007.08.016.

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