# Mechanisms of Anticancer Action of HPMA Copolymer-Bound Doxorubicin

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SUMMARY: This paper summarizes the peculiarities of HPMA copolymer-bound doxorubicin as an anticancer drug. It was found that polymer-bound doxorubicin demonstrated higher anticancer activity compared with free doxorubicin. This phenomenon was explained by the following mechanisms of its anticancer action: preferential accumulation in tumors, internalization in membrane-limited organelles, ability to overcome existing multidrug resistance and not to induce it *de novo*, high intracellular toxicity and inhibition of detoxification enzymes, cell death induction by the activation of specific signaling pathways, and triggering of caspase activation cascades.

## Introduction

*N*-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers have been studied as anticancer drug carriers for several decades[1-8]. Recently, HPMA conjugates with doxorubicin (DOX)[9] and with DOX and N-acylated D-galactosamine (targeting moiety)[10] reached the clinical trial stage. However, the detailed mechanisms of their anticancer activity remain unknown. The main advantage of polymer-based drugs is the preferential accumulation in solid tumors[11,12]. In addition, it was found that a drug bound to the HPMA copolymer backbone was internalized by endocytosis in membrane-limited organelles and released near the nucleus[4]. On the basis of these findings, we hypothesized that HPMA copolymer-bound drugs could activate signaling pathways different from those of the free drug and, therefore, its anticancer efficacy might be different. In addition, they might be more protected from detoxification enzymes and other cellular defensive mechanisms, which could result in the enhanced toxicity. To verify the hypothesis, we synthesized[1] DOX bound via a glycylphenylalanylleucylglycine (GFLG) side chain to an HPMA copolymer (P(GFLG)-DOX, Scheme 1), and studied its efficacy and main mechanisms of action in *in vitro* and *in vivo* experiments[13-19]. The present study summarizes the obtained results and analyzes the main mechanisms of high anticancer activity of HPMA copolymer-bound DOX.

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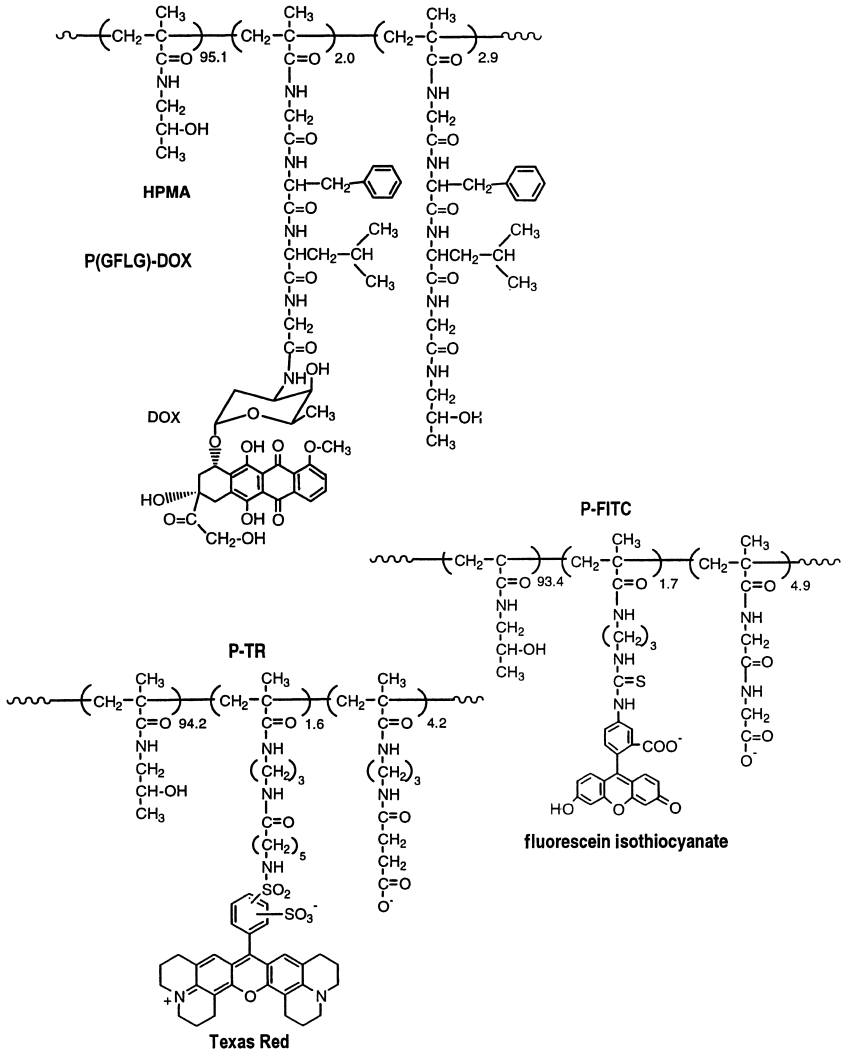


Scheme 1. The conjugates used[1,18]: HPMA copolymer-bound DOX (P(GFLG)-DOX), HPMA copolymer-bound fluorescein isothiocyanate (P-FITC) and HPMA copolymer-bound Texas Red (P-TR).

**Preferential accumulation of HPMA copolymer-bound DOX in solid tumor**

High-molecular-weight water-soluble substances including polymeric anticancer drugs are accumulated preferably in tumors[11,12]. This phenomenon, named the enhanced permeability and retention (EPR) effect, is the result of increased tumor vascular permeability to circulating macromolecules and limited lymphatic drainage. To prove this hypothesis, we

performed experiments on mice bearing xenografts of sensitive (A2780) and DOX-resistant (A2780/AD) human ovarian carcinoma. As expected, P(GFLG)-DOX accumulated mainly in tumors with only minor amounts of the drug found in other organs (Fig. 1).

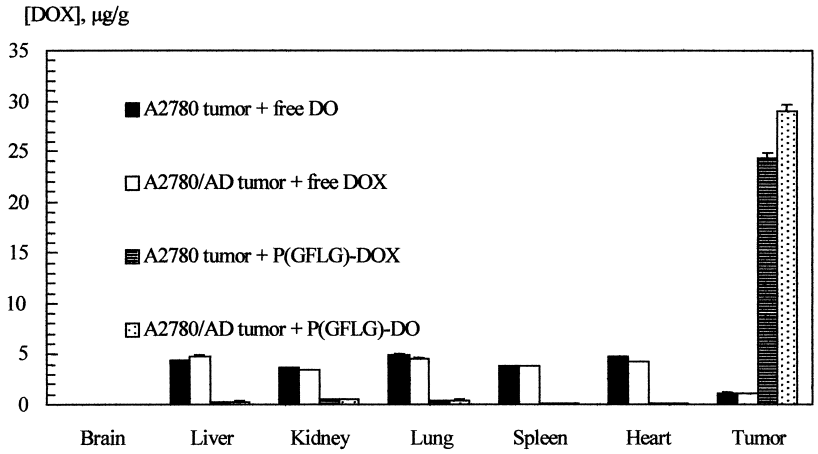


Fig. 1: DOX concentration in tumors and organs as determined by HPLC. (Modified from[16].)

Detailed analysis of drug distribution inside the tumor, however, revealed an unexpected phenomenon. It was found that the distribution of the polymer-bound DOX significantly differed from the same polymer without drug, as well as from the distribution of free DOX (Fig. 2).

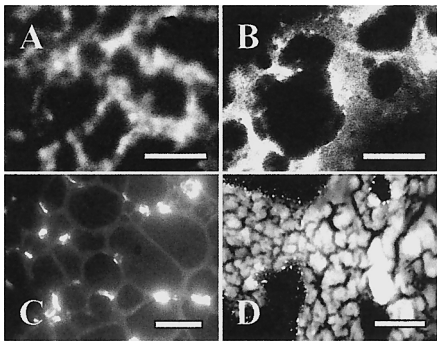


Fig. 2: Typical fluorescent microscope images of tumors from animals treated with HPMA copolymer-bound FITC (A), HPMA copolymer-bound Texas Red (B), free DOX (C) and P(GFLG)-DOX (D). (Scale bars indicate 50 µm; modified from[18].)

The distribution of P(GFLG)-DOX was more homogenous and the mean concentration of the polymeric DOX was significantly higher than that of the free drug[17]. To understand the mechanisms of this phenomenon, we investigated the influence of HPMA copolymer itself, HPMA copolymer-bound DOX and free DOX on vascular permeability of tumor vessels - the

main determinant of the EPR effect. It was found that vascular permeability in tumors was higher than the permeability of normal tissue and correlated with the expression of the gene encoding vascular endothelial growth factor (VEGF), which is responsible for the growth and permeability of vessels (Figs 3, 4). HPMA copolymer labeled with fluorescein isothiocyanate (FITC) and Texas Red (Scheme 1) did not significantly change the expression of the VEGF gene and vascular permeability (data not shown). Administration of free DOX resulted in the overexpression of the VEGF gene and increased vascular permeability in tumor, while the accumulation of P(GFLG)-DOX down-regulated the expression and finally decreased the permeability.

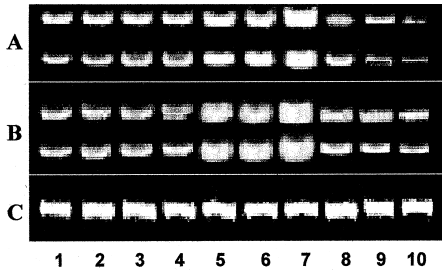


Fig. 3: Expression of two isoforms of VEGF gene in DOX-sensitive (A) and DOX-resistant (B) tumors. Data were obtained by RT-PCR using $\beta_2$-microglobulin as internal standard (C). *1* cells before inoculation; *2-4* untreated tumor; *5-7* tumor treated with free DOX; *8-10* tumor treated with P(GFLG)-DOX; *2,5,8* 18 days; *3,6,9* 25 days; *4,7,10* 32 days. (Modified from[16].)

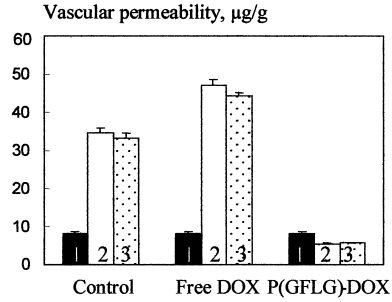


Fig. 4: Vascular permeability in control neighboring tissue (1), DOX-sensitive (2) and DOX-resistant (3) tumors. Vascular permeability was measured by the accumulation of Evans Blue - albumin complex. (Modified from[16].)

These data led us to formulate a concept on the influence of the cytotoxicity of macromolecules on the EPR effect and distribution of anticancer drug through the tumor. The main features of the concept consist of the following. Initially both free DOX and polymer-bound DOX accumulate in the locations with already high vascular permeability. However, the accumulation of these substances produces opposite results. Free DOX increases the permeability, which in turn enhances its accumulation in the same location. This forms a positive feedback leading to the highly inhomogeneous distribution of free DOX in the tumor. In contrast, the P(GFLG)-DOX accumulation decreases the permeability, prevents an additional accumulation in the same location and facilitates the drug entry in different

locations. This forms a negative feedback, which leads to a more homogeneous drug distribution and increases its anticancer activity. Polymers without drug do not change the permeability. As a result, their distribution reflects the initial state of the vascular permeability through the tumor (see Fig. 2).

## Ability to overcome multidrug resistance

Free low-molecular-weight drugs enter cells by diffusion. Initially they accumulate in or near the plasma membrane and therefore are substrates for drug-efflux pumps, mainly P-glycoprotein (encoded by the MDR1 gene) and multidrug-resistance associated protein (MRP). These pumps decrease the concentration of drugs inside the cell and therefore lower their activity (Fig. 5).

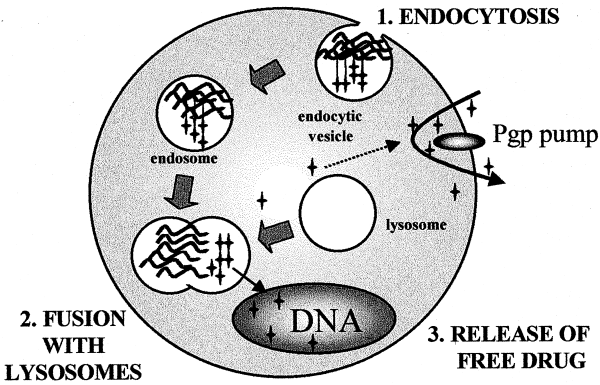


Fig. 5: Mechanisms of the internalization of free and HMPA copolymer-bound drugs.

In contrast, HPMA copolymer-bound drugs enter cells in membrane-limited organelles[4,5]. This decreases their availability for the pumps and also protects them from the action of cellular detoxification mechanisms. It was found that those vesicles finally fuse with lysosomes that accumulate in the perinuclear region[4]. If the drug was bound to the polymer via a lysosomally degradable spacer (GFLG, in the case of HPMA copolymer-bound DOX used in the study), the lysosomal enzymes digest the spacer. The drug escapes the lysosomes and may enter the nucleus. On the basis of this mechanism of P(GFLG)-DOX internalization, we hypothesized that it would overcome existing multidrug resistance and possess higher intracellular toxicity compared with the free drug. To prove this hypothesis, we performed acute and chronic *in vitro* experiments[13,14]. The experimental data showed that, in contrast to

free DOX, P(GFLG)-DOX overcomes the existing MDR1-gene-encoded multidrug resistance and does not induce it *de novo* both after acute and chronic exposure. Figure 6 shows the influence of chronic exposure of sensitive A2780 human ovarian carcinoma cells to free DOX and P(GFLG)-DOX. It can be seen that, after approximately forty days of the experiment, sensitive cells developed MDR1-encoded resistance comparable to A2780/AD DOX-resistant cells. In contrast, the exposure to P(GFLG)-DOX did not significantly change the resistance of the sensitive cells and did not induce multidrug resistance.

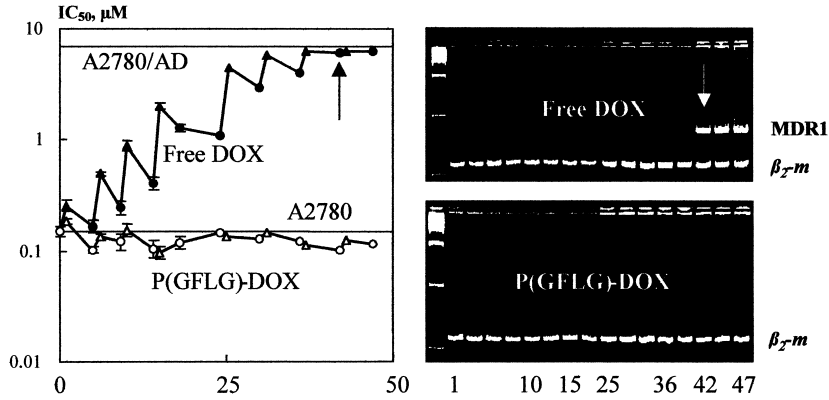


Fig. 6:
Influence of repeated exposure of A2780 human ovarian carcinoma cells to free DOX and P(GFLG)-DOX on their viability. Expression of MDR1 and $\beta_2$-microglobulin ($\beta_2$-m) genes (typical images of gel electrophoresis of RT-PCR products).

The cells were repeatedly exposed to free or P(GFLG)-DOX at 10 or 20% drug concentration necessary to inhibit the growth of the cells by 50 % (IC50). Periods of 48 h of drug exposure (Δ) were followed by 48-96 h recovery periods (O) when cells were grown in fresh media without the drug. The IC50 doses towards free DOX for sensitive A2780 and DOX-resistant A2780/AD cells are shown on the left panel as solid horizontal lines. The arrows on the panels indicate the time interval when the MDR1 gene expression was first detected. The viability was determined as the IC50 dose toward free DOX at time intervals indicated using MTT assay. The gene expression was measured by RT-PCR using $\beta_2$-m as an internal standard. (Modified from[14].)

Another consequence of the internalization of HPMA copolymer-bound DOX in membrane-limited organelles is its high resistance to cellular detoxification. Moreover, we found that P(GFLG)-DOX not only overcomes the action of detoxification enzymes, but it also inhibits them. Figure 7 shows an example of such inhibition. In contrast, free DOX activates the cellular detoxification enzymes. This leads to much higher intracellular toxicity of HPMA

copolymer-bound DOX compared with free DOX. Figure 8 shows that if the $IC_{50}$ dose of drugs was calculated on the basis of their intracellular concentration, it was significantly lower for P(GFLG)-DOX compared with free DOX both in sensitive and resistant cells, which reflects the higher intracellular toxicity of polymer-bound drug.

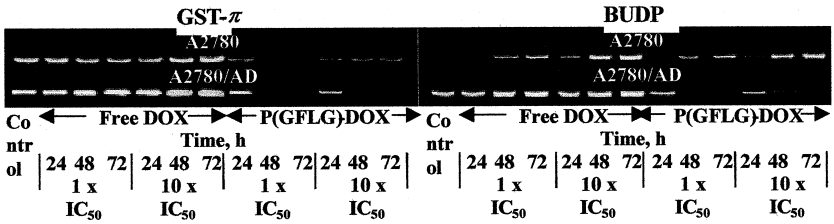


Fig. 7: Influence of the incubation of DOX-sensitive A2780 and DOX-resistant A2780/AD human ovarian carcinoma cells with free DOX and P(GFLG)-DOX on the expression of genes responsible for the drug detoxification. The expression was measured at different times of incubation using RT-PCR. Typical images of gel electrophoresis of PCR products are shown. (Modified from[15].)

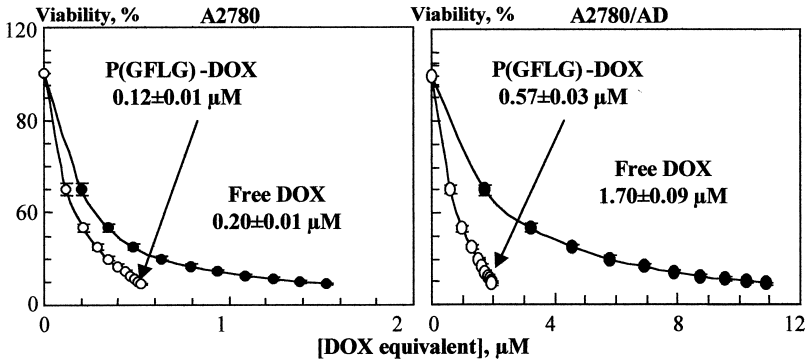


Fig. 8: Cell viability in DOX-sensitive A2780 and DOX-resistant A2780/AD human ovarian carcinoma cells. DOX concentrations in cell lysates were measured by HPLC, the viability was determined using MTT assay. IC50 doses are given. Means ± SD from 4 independent measurements are shown. (Modified from[15].)

The data obtained showed that HPMA copolymer-bound DOX preserved its cytotoxicity during transport in blood and inside the cells. Its distribution in tumor was more uniform when compared with free DOX. On the basis of these facts, we hypothesized that P(GFLG)-DOX should more significantly induce cell death in cancer cells and possibly activate different cell-death-signaling pathways compared with free DOX.

## Cell death induction

The study of signaling pathways of free DOX and P(GFLG)-DOX allows us to formulate common and specific mechanisms of the cell death development under the action of free DOX and HPMA copolymer-bound DOX. Both drugs induced DNA damage. This damage triggers, through the p53 gene, central cell death signal (caspases, protein kinases, etc.), which activates proteases and the c-fos and/or c-jun pathways involved in the activation of endonucleases. In addition, metabolic perturbations, acidosis and activation of lipid peroxidation under the action of DOX, directly or through changes in $Ca^{++}$ ion concentration, might also activate endonucleases. Finally, these processes produce additional DNA damage, which in turn amplifies the cell death signal and forms a vicious circle. The main mechanisms of cell defense against DOX-induced apoptosis include (i) activation of the bcl-2 and HSP-70 gene families, which inhibits proteolysis, lipid peroxidation and limits DNA damage and (ii) mechanisms of DNA repair, synthesis and replication, controlled by thymidine kinases (TK) and topoisomerases (Topo) (Fig.9).

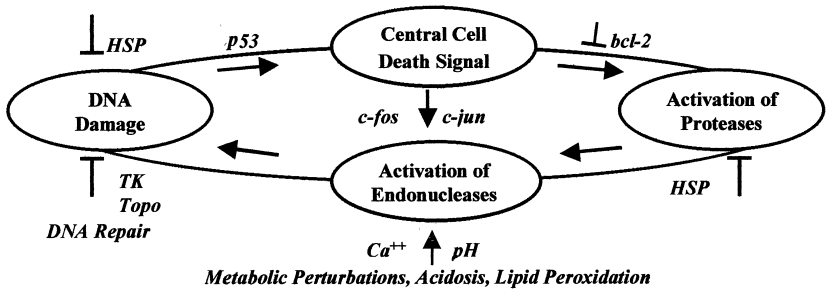


Fig. 9: Main signaling pathways involved in the activation of apoptosis by the action of DOX and P(GFLG)-DOX. (Modified from[17].)

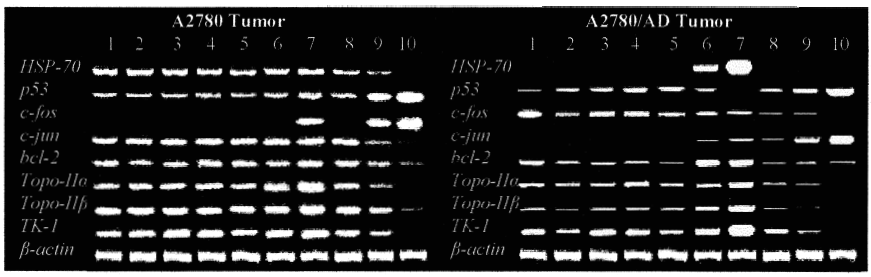


Fig. 10: Typical images of RT-PCR products. *1* cells, *2-4* control tumor, *5-7* free DOX, *8-10* P(GFLG)-DOX. *2,5,8* 18 days, *3,6,9* 25 days, *4,7,10* 32 days of treatment. β-Actin was used as an internal standard.

Analysis of the expression of genes involved in this vicious circle of cell death induction showed the following (Fig. 10). Both free DOX and P(GFLG)-DOX activated the cell death signal through p53, c-fos and/or c-jun pathways. In addition to cell induction mechanisms, free DOX also activated genes involved in the defense against apoptosis and DNA damage (bcl-2, TopoIIα, β and TK-1). In contrast, P(GFLG)-DOX inhibited the mentioned mechanisms of cellular defense against apoptosis. All the mentioned factors led to a more significant cell death induction after the P(GFLG)-DOX action when compared with the free drug (Fig. 11).

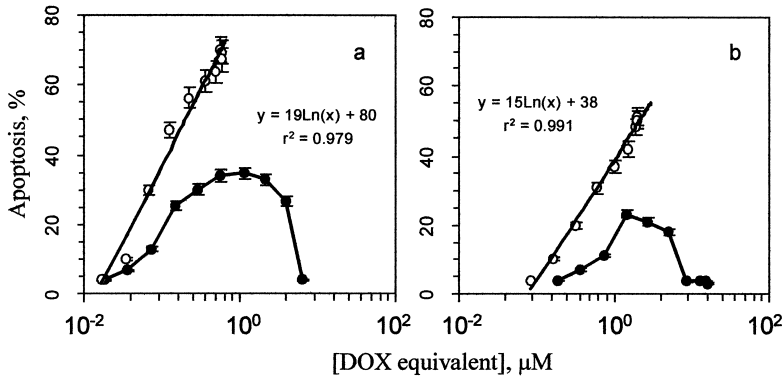


Fig. 11: Concentration-dependent apoptosis induction by free DOX (•) and P(GFLG)-DOX (o) in DOX-sensitive A2780 (a) and DOX-resistant A2780/AD (b) human ovarian carcinoma cells. Percentage of apoptotic cells was determined by flow cytometry after TUNEL labeling. Means ± SD from 4 independent measurements are shown. (Modified from[15].)

This should lead to a higher anticancer activity of HPMA copolymer-bound DOX. The following *in vivo* data support the hypothesis (Fig. 12). We found that free DOX was effective only in sensitive tumors. It did not significantly decrease the tumor size in the resistant tumor,

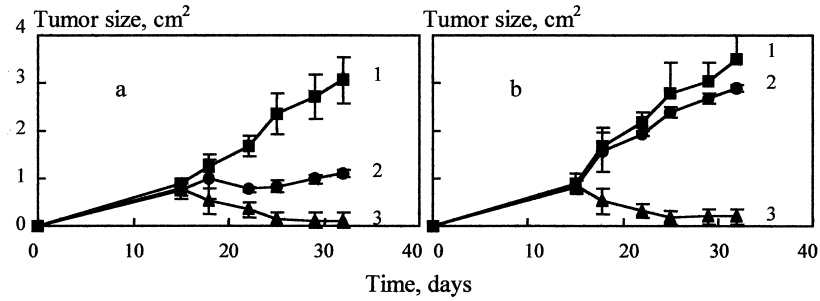


Fig. 12: Effect of free DOX (2) and P(GFLG)-DOX (3) on the size of DOX-sensitive A2780 (a) and DOX-resistant A2780/AD (b) tumors (means ± SD from 3-9 measurements). (Modified from[16].)

whereas cellular defensive mechanisms against drugs were activated. In contrast, P(GFLG)-DOX was equally effective in both sensitive and resistant tumors. This shows that HPMA copolymer-bound DOX effectively overcame drug efflux pumps, cellular detoxification mechanisms and defense against cell death induction. These data also led us to the suggestion that the free DOX and P(GFLG)-DOX might induce different cell death signaling pathways, in particular caspase cascades. Thus the next step of our investigation was aimed at the study of the influence of free and P(GFLG)-DOX on cell death signaling modulated by caspases.

## Activation of specific caspase cascade

Caspases (cystein aspartate specific proteases) are molecular instigators of apoptosis[20,21]. They play a critical role in the execution of the mammalian apoptotic program. On the basis of experimental data we revealed the main pathways involved in apoptosis induction by P(GFLG)-DOX (Fig. 13).

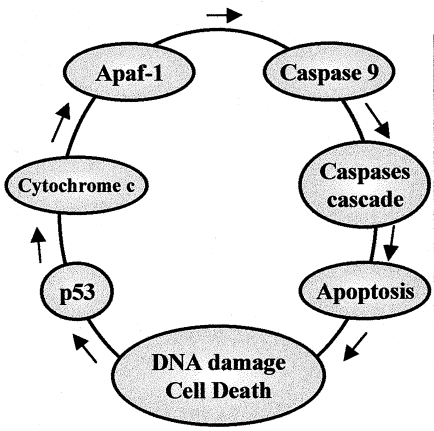


Fig. 13: Signaling cascade of apoptosis induced by free and HPMA copolymer-bound DOX.

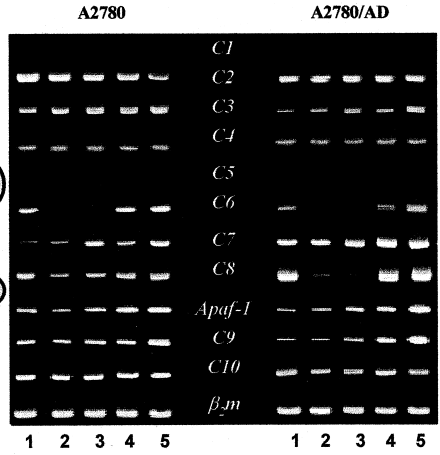


Fig. 14: Typical image of gel electrophoreogram of RT-PCR products. *1* Control, *2,3* free DOX, *4,5* P(GFLG)-DOX, *2,4* 1 x IC50, *3,5* 10 x IC5. C1-C10 - genes encoding human caspases 1-10. $\beta_2$-Microglobulin ($\beta_2$m) was used as an internal standard. (Modified from[19].)

They include the DNA damage produced by DOX directly or through c-fos/c-jun pathways, the p53 gene-dependent central cell death signal, alteration of mitochondrial homeostasis, release of cytochrome c into the cytosol, and Apaf-1-mediated activation of caspase-9. We hypothesized that caspase-9 might trigger other caspase activation events finally resulting in

additional DNA damage and cell death. To verify the hypothesis, we measured the expression of genes encoding human caspases 1-10 (C1-C10) and Apaf-1 under the action of free DOX and P(GFLG)-DOX. The results shown in Fig. 14 revealed the following.

Under all the experimental conditions used, C1 and C5 were not expressed in human ovarian carcinoma cells. In addition, no significant changes were found in the expression of C4, while the expression of genes encoding other caspases was changed after the exposure to free DOX and/or P(GFLG)-DOX. These data corroborate those revealed by Slee et al.[20], which showed that C2, 3, 6, 7, 8, and 10 were processed in response to cytochrome c, and that C1, 4, and 5 failed to be activated under the same conditions. Comparing the present data with known mechanisms of cellular signaling initiated by cytochrome c[20,22] allows us to form a hypothesis about the following signaling pathways of apoptosis (Figs. 15, 16).

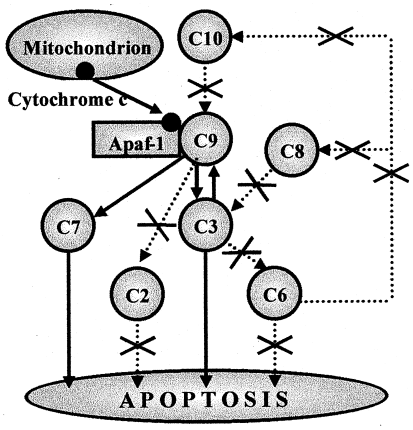


Fig. 15: Signaling pathways of apoptosis induced by free DOX. (Modified from[19].)

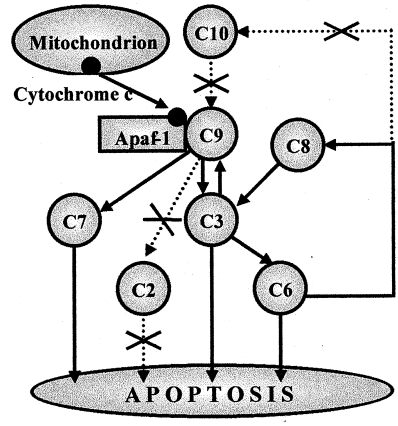


Fig. 16: Signaling pathways of apoptosis induced by P(GFLG)-DOX. (Modified from[19].)

Both drugs induced apoptosis by activating cytochrome c-Apaf1-C9-C3 and cytochrome c-Apaf1-C9- C7 signaling cascades. The degree of activation was more pronounced after the action of P(GFLG)-DOX. In addition, P(GFLG)-DOX activated C6 and C8, producing additional activation of C9 by positive feedback. These data could explain the higher anticancer activity of P(GFLG)-DOX which effectively induced apoptosis by (i) more significant activation of C9-C3 and C9-C7 signaling pathways when compared with free DOX and (ii) triggering an additional C9-C3-C6 pathway and the C9-C3-C6-C8-C9 feedback loop.

## Conclusions

The following main mechanisms lead to the higher antitumor activity of HPMA copolymer-bound DOX compared with free DOX:

- Due to the EPR effect, HPMA copolymer-bound DOX accumulates preferentially in solid tumors while only minor amounts of the drug could be detected in other organs. This decreases the systemic toxicity of P(GFLG)-DOX and increases the DOX concentration in tumor compared with free DOX injected at the same equivalent dose.
- Both free DOX and HPMA copolymer-bound DOX initially accumulate in the regions of tumor with highest vascular permeability. However, an increase in the concentrations of drugs produces opposite results. While free DOX increases the permeability stimulating further drug accumulation in the same location, P(GFLG)-DOX significantly decreases the permeability preventing useless additional accumulation in the same location. This leads to a more uniform distribution of P(GFLG)-DOX within the tumor compared with free DOX.
- A high dose of free DOX activates the MDR1 gene, encoding the P-gp efflux pump, in sensitive cancer cells. In contrast, HPMA copolymer-bound DOX overcomes existing P-gp and multidrug resistance related protein (MRP) pumps in resistant cells and inhibited the expression of the MRP gene both in sensitive and resistant cells. Chronic exposure to free DOX leads to the development of multidrug resistance in sensitive cancer cells. In contrast, P(GFLG)-DOX does not induce multidrug resistance after chronic exposure.
- Free DOX activates cell detoxification mechanisms by increasing the expression of genes encoding glutathione and UDP transferases, while HPMA copolymer-bound DOX suppresses the expression of these genes. This leads to higher intracellular toxicity of P(GFLG)-DOX in comparison with free DOX.
- Both free and conjugated DOX induce apoptosis. However, HPMA copolymer-bound DOX activates apoptosis signaling pathways and, in addition, down-regulates cellular defensive systems against apoptosis. As a result, cell death induction after the action of P(GFLG)-DOX is more pronounced compared with free DOX.
- HPMA copolymer-bound DOX inhibits mechanisms of DNA repair, replication and biosynthesis, while free DOX activates these mechanisms. This suggests that HPMA copolymer-bound DOX produces more DNA damage, which plays a central role in the development of programmed cell death - apoptosis.

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## References

1. J. Kopeček, P. Rejmanová, J. Strohalm, K. Ulbrich, B. Říhová, V. Chytrý, J. B. Lloyd, R. Duncan, U.S. Patent 5,037,883 (1991)
2. R. Duncan, J.K. Coatsworth, S. Burtles, *Human Exp. Toxicol.* **17**, 93 (1998)
3. K. Ulbrich, V. Šubr, J. Strohalm, D. Plocová, M. Jelínková, B. Říhová, *J. Controlled Release* **64**, 63 (2000)
4. B. Říhová, M. Jelínková, J. Strohalm, V. Šubr, D. Plocová, O. Hovorka, M. Novák, D. Plundová, Y. Germano, K. Ulbrich, *J. Controlled Release* **64**, 241 (2000)
5. J. Kopeček, P. Kopečková, T. Minko, Z-R. Lu, *Eur. J. Pharm. Biopharm.* **50**, 61 (2000)
6. V. Omelyanenko, P. Kopečková, R.K. Prakash, C. D. Ebert, J. Kopeček, *Pharm. Res.* **16**, 1010 (1999)
7. Z.-R. Lu, P. Kopečková, J. Kopeček, *Nature Biotechnol.* **17**, 1101 (1999)
8. J.-G. Shiah, Y. Sun, C.M. Peterson, J. Kopeček, *Clin. Cancer Res.* **6**, 1008 (2000)
9. P.A. Vasey, C. Twelves, S.B. Kaye, P. Wilson, R. Morrison, R. Duncan, A. Thomson, L. Murray, T.E. Hilditch, T. Murray, A. Burtles, E. Frigerio, D. Fraier, J. Cassidy, *Clin. Cancer Res.* **5**, 83 (1999)
10. L.W. Seymour, D. R. Ferry, C. Boivin, P. Julyan, J. Doran, M. David, D. Anderson, C. Christodoulou, A.M. Yong, D.J. Kerr, S. Hesslewood, *8th Int. Symp. on Recent Advances in Drug Delivery Systems,* Salt Lake City (UT), 24-27 February 1997, pp.132-135
11. H. Maeda, L.M. Seymour, Y. Miyamoto, *Bioconjugate Chem.* **3**, 351 (1992)
12. Y. Noguchi, J. Wu, R. Duncan, J. Strohalm, K. Ulbrich, T. Akaike, H. Maeda, *Jpn. J. Cancer Res.* **89**, 307 (1998)
13. T. Minko, P. Kopečková, V. Pozharov, J. Kopeček, *J. Controlled Release* **54**, 223 (1998)
14. T. Minko, P. Kopečková, J. Kopeček, *J. Controlled Release* **59**, 133 (1999)
15. T. Minko, P. Kopečková, J. Kopeček, *Pharm. Res.* **16**, 986 (1999)
16. T. Minko, P. Kopečková, J. Kopeček, *Int. J. Cancer* **86**, 108 (2000)
17. T. Minko, P. Kopečková, J. Kopeček, *9th Int. Symp. on Recent Advances in Drug Delivery Systems,* Salt Lake City (UT), 24-27 February 1999, pp. 215-216.
18. T. Minko, P. Kopečková, V. Pozharov, K. Jensen, J. Kopeček, *Pharm. Res.* **17**, 505 (2000)
19. T. Minko, P. Kopečková, J. Kopeček, *J. Controlled Release*, in press
20. E.A. Slee, M.T. Harte, R.M. Kluck, B.B. Wolf, C.A. Casiano, D.D. Newmeyer, H.-C. Wang, J.C. Reed, D.W. Nicholson, E.S. Alnemri, D.R. Green, S. J. Martin, *J. Cell Biol.* **144**, 281 (1999)
21. S. Hu, S.J. Snipas, C. Vincenz, G. Salvesen, V.M. Dicit, *J. Biol. Chem.* **237,** 29648 (1998)
22. P.Villa, S.H. Kaufmann, W.C. Earnshaw, *Trends Biochem. Sci.* **22,** 388 (1997)