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Research paper

HPMA copolymer–anticancer drug–OV-TL16 antibody conjugates. 3. The effect of free and polymer-bound Adriamycin on the expression of some genes in the OVCAR-3 human ovarian carcinoma cell line

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Abstract

The effect of an *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer–Adriamycin–OV-TLl6 antibody conjugate [P(GFLG)–ADR–Ab] on OVCAR-3 human ovarian carcinoma cells was studied. A nontargeted HPMA copolymer–ADR conjugate (P(GFLG)–ADR) and free ADR were the controls. The IC_{50} doses were 0.65, 3.0, and 65 μM for free ADR, targeted P(GFLG)–ADR–Ab conjugate, and nontargeted P(GFLG)–ADR conjugate, respectively. These differences reflect the different mechanisms of cell entry of the compounds evaluated. Free ADR and HPMA copolymer–ADR conjugates had different impacts on the expression of *MDR1*, *MRP*, c-*fos*, c-*jun*, and *bcl*-2 genes which encode the P-glycoprotein (*MDR1*) and the multidrug resistance-associated protein (*MRP*) efflux pumps, and play an important role in cell death signaling pathways (c-*fos*, c-*jun*, and *bcl*-2). Whereas high doses of free ADR induced *MDR1* gene expression, HPMA copolymer-bound ADR appeared to be without effect. On the contrary, expression of the *MRP* gene was not influenced by free ADR, whereas HPMA copolymer–ADR conjugates seemed to suppress the gene expression in a concentration-dependent manner. There were differences in the expression of c-*fos*, c-*jun*, and *bcl*-2 genes after the incubation of OVCAR-3 cells with free and HPMA copolymer-bound ADR indicating differences in activation of cell death signaling pathways. © 2000 Published by Elsevier Science B.V. Published by Elsevier Science Ltd.

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1. Introduction

N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers were developed as macromolecular carriers of anticancer drugs. The biorecognition of such conjugates by a subset of cells may be enhanced by the attachment of antibodies, saccharides, hormones (reviewed in [1]), or synthetic receptor-binding epitopes [2]. Recently, the activity of conjugates containing a monoclonal OV-TL16 antibody (Ab) was evaluated [3]. The Ab has a high affinity to an antigen associated with numerous human ovarian carcinomas as well as the human ovarian carcinoma cell line OVCAR-3 [4].

Multidrug resistance (MDR) is one of the major causes for failure of ovarian cancer treatment. It is well established that macromolecular drug delivery systems have the potential to overcome MDR. We have shown recently that an HPMA copolymer–Adriamycin (ADR) conjugate may, at least partially, avoid the ATP driven P-glycoprotein (Pgp) efflux pump in A2780/AD multidrug resistant human ovarian carcinoma cells [5]. Moreover, in contrast to free ADR, chronic exposure to HPMA copolymer–ADR conjugate did not induce MDR in A2780 sensitive human ovarian carcinoma cells [6]. However, in both studies nontargeted HPMA copolymer–ADR conjugates were used. Consequently, the IC_{50} doses of free and copolymer-bound ADR in A2780 sensitive cells differed substantially due to the different mechanisms of cell entry of the low and high molecular weight substrates [5].

The aim of this study was threefold. Firstly, to compare the effect of free and HPMA copolymer-bound ADR on the expression of multidrug resistance related genes by using a conjugate and protocol where the IC_{50} doses of free and copolymer-bound ADR were closer than in previous studies. Secondly, to verify the results obtained with A2780 cells in a different (OVCAR-3) human ovarian carci-

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noma cell line, and thirdly to compare the effect of free ADR and HPMA copolymer-ADR–OV–TL–16 antibody conjugate on the expression of several genes associated with cell death.

To this end, we have synthesized an HPMA copolymer–ADR–OV-TLl6 antibody conjugate and studied its effect on the OVCAR-3 human ovarian carcinoma cell line. Simultaneous measurements of cell viability and of the *MDR1*, *MRP*, c-*fos*, c-*jun*, and *bcl*-2 gene expression were performed. An HPMA copolymer–ADR conjugate was used as a control.

2. Materials and methods

The human cancer cell line NIH: OVCAR-3 was purchased from American Type Culture Collection. Cells were cultured in RPMI 1640 medium (Sigma) supplemented with 20% fetal bovine serum (HyClone) and 10 mg/ml insulin (Sigma). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 (v/v) in air. The OV-TL16 secreting hybridoma cells were grown as ascites in pristaned Balb/c mice (Charles River). The OV-TLI6 antibody (Ab) was isolated by ammonium sulfate precipitation followed by affinity chromatography on a Gamma Bind Plus column (Pharmacia).

The polymer precursors and the targeted [P(GLFG)–ADR–Ab)] and nontargeted [P(GFLG)–ADR] HPMA copolymer conjugates were synthesized based on previously described procedures [7,8].

The polymer precursors 1 and 3 (Table 1) were prepared by radical precipitation copolymerization of HPMA with *N*-methacryloylglycylphenylalanylleucylglycine *p*-nitrophenyl ester (MA–GFLG–ONp) in acetone at 50°C for 48 h using 2,2′-azobisisobutyronitrile (AIBN) as the initiator. The molar ratio of monomers in the polymerization mixture for the polymer precursor 1 was 94:6 (HPMA:MA–GFLG–ONp) and for polymer precursor 3 the ratio was 88:12. The copolymers were purified by precipitation from methanol into an excess of acetone/ether (3:1).

Polymer precursor 4 was prepared by reacting polymer precursor 3 with ADR.HCl in DMS0 in the presence of triethylamine (mole ratio ONp:ADR:Et_3N = 1 : 0.4 : 0.4). The reaction mixture was stirred at room temperature for 1 h. The polymer was precipitated into acetone/ether (3:1) and further purified on a Sephadex LH-20 column using MeOH/l% CH_3COOH as the eluent. The polymer fraction was collected, methanol evaporated and the polymer precursor 4 isolated by re-precipitation into acetone/ether (3:1).

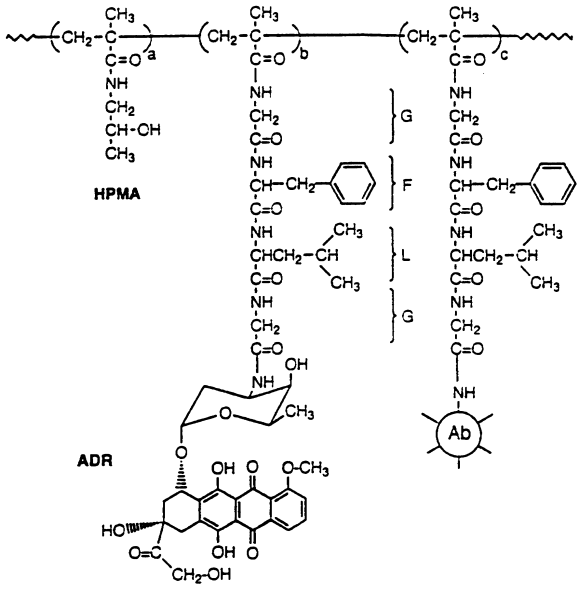
The non-targeted conjugate 2, P(GFLG)–ADR, was prepared by aminolysis of polymer precursor 1 with ADR.

The targeted conjugate 5, P(GFLG)–ADR–Ab, was synthesized by the reaction of polymer precursor 4 with the monoclonal OV-TL16 antibody. The Ab was attached

Table 1
Characterization of polymer precursors[a] and HPMA copolymer conjugates

Polymer no.	Structure	Mol% of side-chains	μmol ligand per mg polymer (or conjugate)	Ab:Polym.:ADR (wt.%)	Molecular weight
1	P–GFLG–ONp (polymer precursor)	5.1	0.309 (ONp)	NA[b]	22 500[c,d] (M_w/M_n = 1.5
2	P–GFLG–ADR (nontargeted conjugate)	2.4	0.149 (ADR)	0:91.9:8.1	24 000[c]
3	P–GFLG–ONp (polymer precursor)	8.7	0.481 (ONp)	NA	19 600[c,d] (M_w/M_n = 1.3)
4	P–(GFLG)–ADR–ONp (polymer precursor)	2.9	0.150 (ADR)	0:91.7:8.3	20 500[c,d]
		4.7[e]	0.242 (ONp)		
5	P–(GFLG)–ADR–Ab (targeted conjugate)	NA	0.0589 (ADR)	60.6:36.7:3.3[f]	249 000[g]
			0.000389 (Ab)		

[a] HPMA copolymers containing *p*-nitrophenoxy (ONp) reactive ester groups.
[b] Not applicable.
[c] Molecular weight average (M_w) and polydispersity (M_w/M_n) of polymer was estimated by SEC using the FPLC system, Superose 12 column, buffer PBS + 30% (vol) acetonitrile, calibrated with poly(HPMA) fractions.
[d] Determined after aminolysis with 1-amino-2-propanol.
[e] Determined as released ONp (400 nm) after hydrolysis in 0.1 N NaOH.
[f] Determined from ADR content and protein content by Lowry (molecular ratio of Ab:polymer:ADR = 1:5:15).
[g] Calculated from the composition of polymer (weight ratio of Ab and ADR containing HDMA copolymer) and SEC profile.



Scheme 1. Structure of HPMA copolymer-ADR–OV–TL16 (P(GFLG)–ADR–Ab) conjugate.

to polymer precursor 4 by aminolysis in an aqueous (buffer) solution. The HPMA copolymer–ADR–OV-TL16 conjugate is referred to as P(GFLG)–ADR–Ab. The structure of the latter is shown in Scheme 1, and the characterization of polymer precursors and conjugates is shown in Table 1. The nontargeted (control) HPMA copolymer–ADR conjugate is referred to as P(GFLG)–ADR.

Cytotoxicity assay, RNA preparation, cDNA synthesis, analysis of *MDRI*, *MRP*, c-*fos*, c-*jun*, and *bcl*-2 gene expression by reverse transcription polymerase chain reaction (RT–PCR) were performed as previously described [5,9].

3. Results and discussion

HPMA copolymer–anticancer drug conjugates have shown activity toward numerous cancer models (reviewed in [1]). The observation that they may be able to overcome *MDR1* gene-encoded resistance in A2780/AD resistant human ovarian carcinoma cells [5] and not induce MDR during chronic exposure to A2780 sensitive cells in vitro [6] bodes well for their potential in ongoing clinical trials.

In previous experiments on A2780 cells the biological properties of free and HPMA copolymer-bound ADR were compared [5,6]. Due to the difference in the mechanism of cell uptake, the IC_{50} doses of free and HPMA copolymer-bound ADR were substantially different. Consequently, the comparison of free and high molecular weight ADR at doses related to their individual IC_{50} doses was marred by the possibility that the high concentration of the HPMA copolymer–ADR conjugate may have resulted in additional mechanisms of drug action.

To clarify this question, we have synthesized an HPMA copolymer–ADR–OVTL16 antibody conjugate (Table 1) and compared its activity toward OVCAR-3 human ovarian carcinoma cells with a (nontargeted) HPMA copolymer–ADR conjugate and free ADR. The OV-TL16 antibody is complementary to the OA-3 antigen expressed on OVCAR-3 cells [4].

The IC_{50} doses (Fig. 1, Table 2) clearly indicate the different mechanisms of uptake of low- and high-molecular weight drugs. The IC_{50} dose of the nontargeted P(GFLG)–ADR conjugate was 20 times higher when compared to the targeted P(GFLG)–ADR–Ab conjugate. The lowest IC_{50} dose, as expected, was observed with free ADR. The latter was 100 times lower than the IC_{50} dose of the nontargeted conjugate, but only five times lower than the targeted conjugate. Biorecognition of the targeted conjugate decreased the IC_{50} dose substantially, due to the internalization by receptor-mediated endocytosis (pinocytosis), when compared to the nontargeted conjugate, which was internalized by fluid-phase pinocytosis, a slow process indeed. After reaching the lysosomal compartment, ADR is cleaved from both conjugates by thiol proteinases [1] and diffuses via the cytoplasm into the cell nucleus [3]. The GFLG side-chain, used in this study, was designed to fit the active site of cathepsin B, the most important enzyme in the lysosomal cleavage of the HPMA copolymer-drug conjugates [1]. Consequently, the cleavage site in P(GFLG)–ADR and P(GFLG)–ADR–Ab is the bond originating in the terminal glycine residue resulting in the release of unmodified Adriamycin [10].

MDR in model systems is usually conferred by two different integral membrane proteins, the 170 kDa P-glycoprotein (Pgp) and the 190 kDa multidrug resistance-associated protein (MRP). They are both members of the ATP-binding cassette (ABC) superfamily of transport proteins. The expression of *MDR1* and *MRP* genes, which encode these

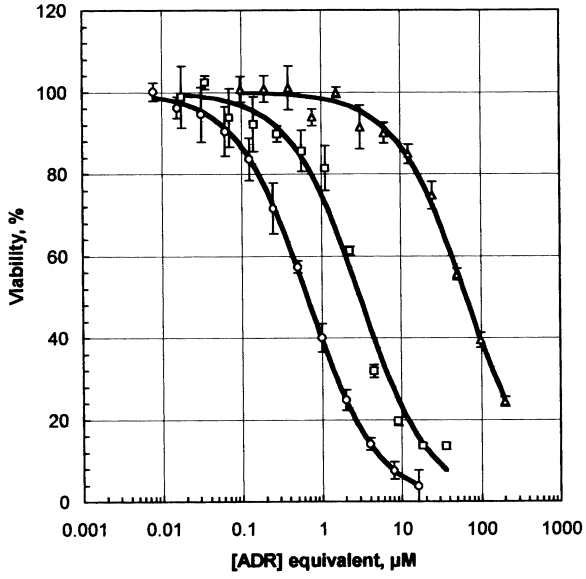


Fig. 1. Viability of OVCAR-3 cells towards free ADR (circles), P(GFLG)–ADR–Ab (squares) and P(GFLG)–ADR (triangles). Means ± SD are shown.

Table 2
IC_{50} doses (means ± SD) for OVCAR-3 cells incubated with free ADR and HPMA copolymer-bound ADR

Sample	IC_{50} (μM)
Free ADR	0.654 ± 0.051
P(GFLG)–ADR–Ab	3.01 ± 0.12*
P(GFLG)–ADR	65.2 ± 4.62**

*$P < 0.05$ when compared with free ADR.
**$P < 0.05$ when compared with P(GFLG)–ADR.

glycoproteins, can be modified by transient exposure to anticancer drugs.

We have used quantitative RT–PCR to determine *MDR1* and *MRP* gene expression. Untreated OVCAR-3 cells did not express a detectable amount of the *MDR1* gene, however, the *MRP* gene was expressed (Fig. 2). OVCAR-3 cells were incubated 72 h with the IC_{50} dose and the 10 times IC_{50} dose of all three drugs tested, i.e. free ADR, P(GFLG)–ADR, and P(GFLG)–ADR–Ab. The expression of the *MDR1* gene was induced only in cells incubated with the 10 times IC_{50} dose of free ADR (Fig. 2). On the contrary, the expression of the *MRP* gene was not influenced by incubation with free ADR (Fig. 2).

However, both targeted and nontargeted HPMA copolymer–ADR conjugates inhibited the *MRP* gene expression. Incubation with an IC_{50} dose of P(GFLG)–ADR or P(GFLG)–ADR–Ab decreased the *MRP* gene expression about three times, whereas incubation with the 10 times IC_{50} dose suppressed the *MRP* gene expression below the detection limit (Fig. 2). The fact that the MRP protein can effectively efflux Adriamycin seems to indicate the possibility that ADR released from the HPMA copolymer would less likely be effluxed from the cell interior due to the decrease of the *MRP* gene expression.

To further analyze the differences in the mechanism of action of free and HPMA copolymer-bound ADR we measured the expression of c-*fos*, c-*jun*, and *bcl*-2 genes, which take part in numerous cellular functions, including cell death signaling pathways and cell resistance to various drugs. In particular, it was shown that an up-regulation of c-*fos*, c-*jun* genes in most cases activates apoptosis, while the overexpression of the *bcl*-2 gene increases the cell resistance to many damaging exogenous factors including anthracyclines ([9] and references therein). The results of the RT–PCR analysis (Fig. 2) demonstrated that the expression of the c-*fos* gene in OVCAR-3 cells was under the detection threshold of the method used (less then 5% of the β_2m mRNA level). Incubation of OVCAR-3 cells with $1 \times IC_{50}$ dose of free ADR did not activate the c-*fos* gene, while the $10 \times IC_{50}$ dose caused its overexpression up to about 40% relative to the expression of β_2m. In contrast, both HPMA copolymer–ADR conjugates (2 and 5, Table 1) significantly increased the expression of c-*fos* gene at both concentrations used. Further analysis showed that free ADR led to a statistically significant ($P < 0.05$) increase in the c-

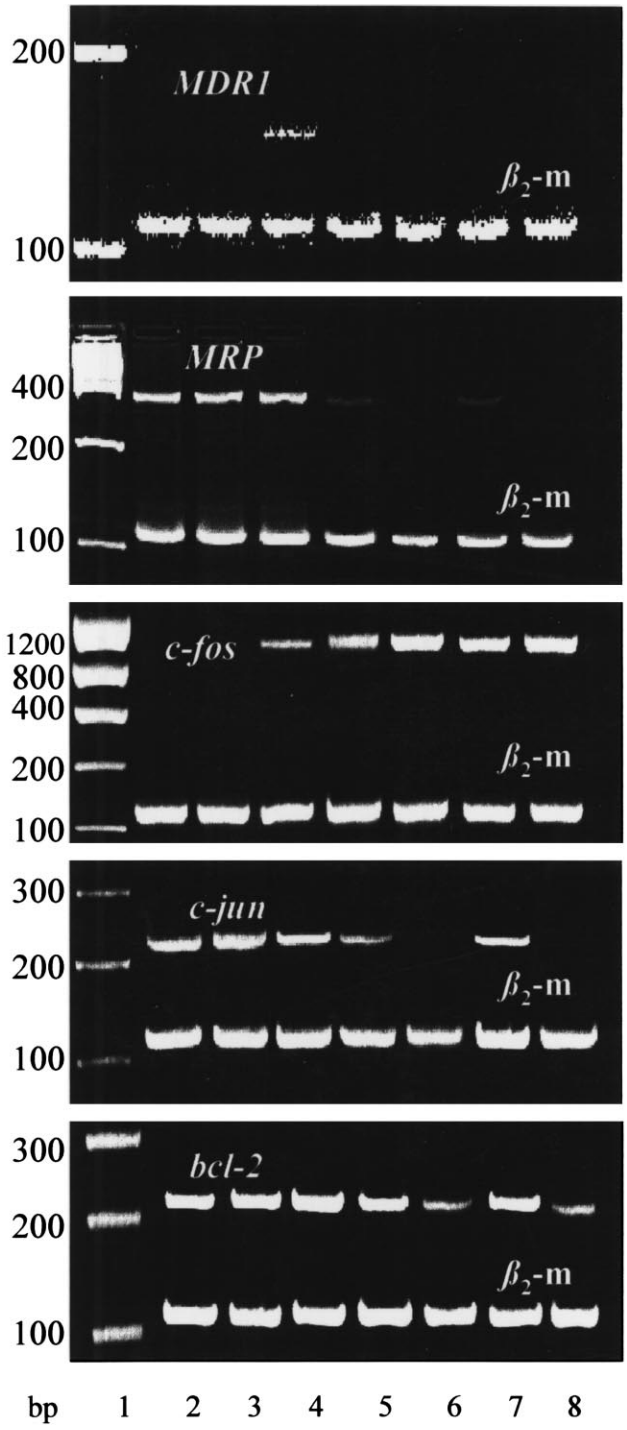


Fig. 2. *MDR1*, *MRP*, c-*fos*, c-*jun*, and *bcl*-2 gene expression in OVCAR-3 cells. The expression was estimated using RT–PCR. PCR products were subjected to electrophoresis in 2% (c-*fos*) or 4% (*MDR1*, *MRP*, c-*jun*, *bcl*-2) MetaPhor agarose gel. The following samples were loaded into the gel: Line 1, DNA ladder; line 2, OVCAR-3 cells (control); line 3, OVCAR-3 cells incubated 72 h with free ADR ($1 \times IC_{50}$); line 4, OVCAR-3 cells incubated 72 h with free ADR ($10 \times IC_{50}$); line 5, OVCAR-3 cells incubated 72 h with P(GFLG)–Ab–ADR ($1 \times IC_{50}$); line 6, OVCAR-3 cells incubated 72 h with P(GFLG)–Ab–ADR ($10 \times IC_{50}$); line 7, OVCAR-3 cells incubated 72 h with P(GFLG)–ADR ($1 \times IC_{50}$); line 8, OVCAR-3 cells incubated 72 h with P(GFLG)–ADR ($10 \times IC_{50}$).

jun gene expression at the $1 \times IC_{50}$ dose and to the decrease in expression when cells were incubated with the $10 \times IC_{50}$ dose. On the other hand, both HPMA copolymer–ADR conjugates down-regulated the c-*jun* gene expression to non-detectable levels. In addition, while free ADR activated the expression of the *bcl*-2 gene, the HPMA copolymer–ADR conjugates down-regulated the expression. We have shown previously that activation of the c-*fos* signaling pathway associated with down-regulation of c-*jun* and *bcl*-2 genes led to an induction of apoptosis in A2780 ovarian carcinoma cells, and the degree of apoptosis correlated with the expression of these genes [9]. In most cases an activation of c-*fos* pathway increased the degree of apoptosis, while an activation of c-*jun* and *bcl*-2 pathways increased the resistance of ovarian carcinoma cells to ADR [9]. The fact that incubation of OVCAR-3 cells with both HPMA copolymer-ADR conjugates 2 and 5 resulted in a decreased *bcl*-2 gene expression suggests the enhanced therapeutic potential of polymeric forms of ADR when compared to free ADR.

The data seem to indicate that the difference in MDR-related biological activity of HPMA copolymer–ADR conjugates (when compared to free ADR) is based on their lysosomotropism. In the conjugates studied, ADR was bound to the HPMA copolymer via the lysosomally degradable GFLG oligopeptide side-chain. After internalization by receptor-mediated endocytosis, ADR is released and diffuses out of the lysosomal compartment into the perinuclear region [1,3]. Consequently, the probability of its interaction with nuclear DNA may be higher than for the free drug. In addition, the incubation of OVCAR-3 cells with free and HPMA copolymer-bound ADR resulted in different impact on the c-*fos*, c-*jun*, and *bcl*-2 gene expression indicating the involvement of different signaling pathways. Finally, the different subcellular localization of conjugates when compared to free drug may result in the modification of the cytotoxicity of the drug cleaved from the lysosomally degradable GFLG side-chains.

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