Comparison of the Anticancer Effect of Free and HPMA Copolymer-Bound Adriamycin in Human Ovarian Carcinoma Cells

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Purpose. To study peculiarities and the mechanism of the anticancer effect of free and HPMA copolymer-bound ADR in sensitive and resistant human ovarian carcinoma cells.
Methods. Sensitive A2780 and ADR resistant A2780/AD cells were exposed to different doses of drugs during 12, 24, 36, 48, 60, and 72 hours. Cell viability, drug accumulation, apoptosis, cellular metabolism, lipid peroxidation, DNA content and gene expression were studied.
Results. HPMA copolymer-bound ADR (P(GFLG)-ADR) possessed a comparable cytotoxicity to free ADR when comparison was based on intracellular concentrations. While free ADR up-regulated genes encoding ATP driven efflux pumps (*MDR1, MRP*), P(GFLG)-ADR overcame existing pumps and down regulated the *MRP* gene. Free ADR also activated cell metabolism and expression of genes responsible for detoxification and DNA repair. P(GFLG)-ADR down-regulated *HSP-70, GST-π, BUDP, Topo-IIα*, β, and *TK-1* genes. Apoptosis, lipid peroxidation and DNA damage were significantly higher after exposure to P(GFLG)-ADR, as reflected by simultaneous activation of *p53, c-fos* in A2780 cells) or *c-jun* (A2780/AD) signaling pathways and inhibition of the *bcl-2* gene. Differences between free ADR and P(GFLG)-ADR increased with the time of incubation and drug concentration.
Conclusions. P(GFLG)-ADR overcame drug efflux pumps, more significantly induced apoptosis and lipid peroxidation, inhibited DNA repair, replication, and biosynthesis when compared to free ADR.

KEY WORDS: adriamycin; doxorubicin; HPMA copolymer; apoptosis, multidrug resistance; gene expression; signal transduction.

INTRODUCTION

Multidrug resistance is one of the main reasons for the failure of chemotherapy to treat the majority of cancers. Drug resistance may be intrinsic or acquired as a consequence of drug exposure. The expression of ATP-dependent efflux pumps, including membrane bound P-glycoprotein (Pgp) and multidrug resistance-associated protein (MRP), results in the decrease of intracellular drug concentrations. Hydrophobic low molecular weight drugs, such as ADR, are substrates for efflux pumps. Consequently, their intracellular concentration decreases with increased cell resistance (1–3).

In contrast, polymer-bound drugs enter cells by endocytosis in membrane-limited organelles which ultimately fuse with lysosomes to form secondary lysosomes. This subcellular trafficking pathway permits the release of the drug from the polymer carrier in the lysosomal compartment followed by its diffusion into the cytoplasm, providing that the drug was bound to the carrier via a lysosomally degradable spacer (4). In addition, it was found that secondary lysosomes (containing P(GFLG)-ADR) accumulate in the perinuclear region (5). Consequently, it appears that polymeric drugs have a potential to overcome the action of active efflux pumps located in the plasma membrane (6,9). Recently we revealed that, in contrast to free ADR, HPMA copolymer-bound ADR not only overcame an existing *MDR1* encoded multidrug resistance in human ovarian carcinoma cells (8), but did not induce it *de novo* (9). Similar results were obtained with OV-TL16 antibody-targeted HPMA copolymer conjugates on human ovarian carcinoma OVCAR-3 cells (10).

We hypothesize that P(GFLG)-ADR, being internalized in membrane-limited organelles and released in the perinuclear region, could activate different signaling pathways than free ADR and therefore its anticancer effect might be different. As a result, P(GFLG)-ADR might be more protected from detoxification mechanisms, resulting in an enhanced activation of apoptosis, lipid peroxidation and DNA damage when compared to free ADR. The present study was aimed at verifying this hypothesis.

MATERIAL AND METHODS

Drugs

Adriamycin was a kind gift from Dr. A. Suarato, Pharmacia-Upjohn, Milano, Italy. The HPMA copolymer-bound ADR (P(GFLG)-ADR; P is the HPMA copolymer backbone) was synthesized as previously described (11,12). A lysosomally degradable glycylphenylalanylleucylglycine (GFLG) spacer was used as the oligopeptide side chain (Scheme 1). The conjugate was synthesized using a two step procedure. In the first step, the polymer precursor was prepared by radical precipitation copolymerization of HPMA and N-methacryloylglycylphenylalanylleucylglycine p-nitrophenyl ester (12). The polymer precursor contained 5.5 mol% active ester groups ($M_w = 22{,}000$, $M_w{:}M_n = 1.5$). ADR was bound to the polymer precursor by aminolysis (13). The conjugate was purified on a Sephadex LH 20 (Pharmacia) column using methanol containing 10% DMSO and 1% CH_3COOH as the eluent. The P(GFLG)-ADR conjugate contained 5 wt.-% (1.6 mol%) of ADR.

All concentrations of P(GFLG)-ADR were expressed in ADR equivalents. Drug solutions were sterilized by filtering through a 0.2-μm filter prior to use.

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ABBREVIATIONS: ADR, adriamycin (doxorubicin); dUTP, deoxyuracil triphosphate; HPMA, N-(2-hydroxypropyl)methacrylamide; IC_{50} dose, drug concentration which inhibits growth by 50% relative to non-treated control cells; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P(GFLG)-ADR, HPMA copolymer-bound ADR (P is the HPMA copolymer backbone; GFLG, the lysosomally degradable glycylphenylalanylleucylglycine spacer); Pgp, P-glycoprotein; TBA, thiobarbituric acid; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling.

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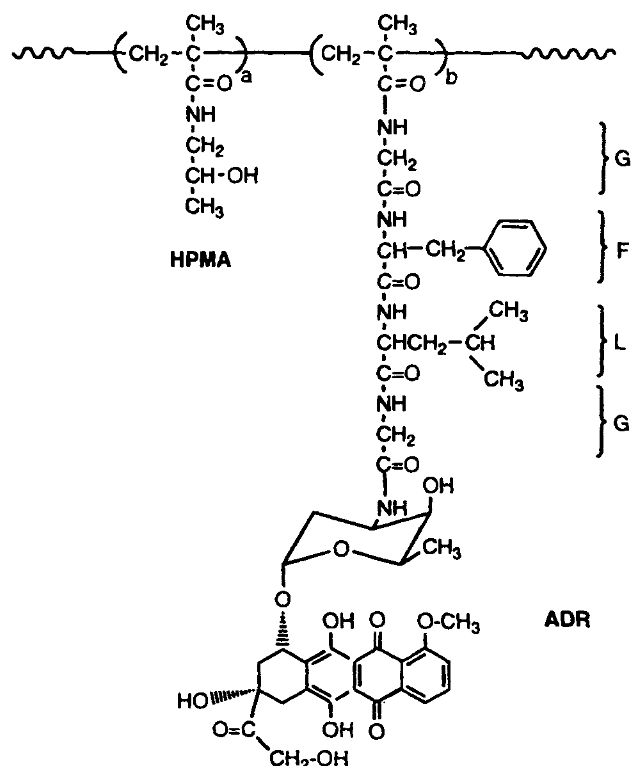


Schematic 1. Structure of HPMA copolymer-adriamycin conjugate (P(GFLG)-ADR).

Cell Lines

The human ovarian carcinoma cell lines, A2780 (sensitive) and A2780/AD (ADR resistant) were obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center, PA). A2780/AD is a Pgp-expressing cell line (8,9,14). Cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (HyClone), 10 μg/ml insulin (HyClone), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 (v/v) in air.

Cytotoxicity Assay

The cytotoxicity of drugs was assessed using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (15). The results of the cytotoxicity assay were used for the calculation of the IC_{50} dose (drug concentration which inhibits growth by 50% relative to non-treated control cells) after incubation with free ADR and P(GFLG)-ADR as previously described (8).

Adriamycin Accumulation

A2780 and A2780/AD cells were exposed to different concentrations of ADR and P(GFLG)-ADR for 72 h. At the end of the incubation period, the medium and 1×10^7 cells were separated by centrifugation. The concentration of ADR in the cell lysate and in the medium was determined by an HPLC assay. The ADR amount determined in the cell lysate was the sum of the surface bound and intracellularly located drug. The amount of ADR in the medium allowed for a check of mass balance.

Cells were separated from the media by centrifugation and lyzed with a protease containing lysis buffer from QIAamp kit for DNA isolation (Qiagen) and centrifuged. The samples were subjected to 2 M HCl acid hydrolysis for 10 min at 85°C prior to neutralization, spiked with daunomycin (internal standard), and centrifuged after silver nitrate (0.1 N) precipitation. The HPLC analysis was performed on a C18 column using the Dionex BioLC chromatograph equipped with an FD-300 fluorescence detector (excitation 480 nm, emission 560 nm). A 10:20:70 v/v/v solution of methanol:isopropanol:glycine buffer (10 mM, pH 2.6) was used as the mobile phase.

Cell Metabolism

To estimate the intensity of total cellular metabolism, the rate of cellular glucose uptake was measured. Glucose concentration in the fresh cell media and after incubation of cells with or without drug was measured using an enzymatic glucose oxidase colorimetric end-point kit (No. 510-DA, Sigma). The glucose uptake was calculated as the amount of glucose consumed per unit of time per cell.

To characterize the cell anaerobic metabolism, the concentration of lactic acid in the media was measured by an enzymatic assay kit (No. 735-10, Sigma). Aerobic metabolism was analyzed by the measurement of mitochondrial activity per cell expressed as amount of formazan formed (in terms of optical density) per unit of time per cell (9,16).

Analysis of Gene Expression by Quantitative RT-PCR

Combination of reverse transcription (RT) and quantitative polymerase chain reaction (PCR) was used for the quantitative analysis of gene expression in cells incubated with drugs for 24, 48, and 72 h. Total cellular RNA was isolated using an RNeasy kit (Qiagen) and a QIAshredder micro spin homogenizer (Qiagen). First-strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (Pharmacia) according to the manufacturer's instructions with 1 μg of total cellular RNA (from 1×10^7 cells) and 100 ng of random hexadeoxynucleotide primer (Pharmacia). After synthesis, the reaction mixture was diluted 1:3 with water and immediately subjected to PCR.

PCR was carried out using an Air Thermocycler (Idaho Technology) with the diluted first-strand reaction mixture, 1 unit of Taq Polymerase (GibcoBRL), and 0.5 μM of specific primers in a final volume of 50 μl. The pairs of primers used to amplify each type of cDNA and the PCR regimes are detailed in Table 1. Agarose gel electrophoresis was used for the separation of PCR products. The gels were stained with ethidium bromide, photographed by a digital camera connected to a computer and scanned. Gene expression was calculated as the ratio between the amount of PCR product (area under the curve in the gel scan) corresponding to the mRNA of the gene of interest and those of the internal standard (β-*actin*). Our previous experiments [10] showed that the calibration curve for the method was linear within a 40-fold range from 5 to 200 ng of PCR products corresponding to 2–80 mRNA copies per cell.

Apoptosis Detection

Two methods were used to estimate the apoptotic activity in cells (1×10^7) treated by free ADR and P(GFLG)-ADR.

Table 1. List of Primers (from 5′ to 3′) and PCR Regimes Used for RT-PCR.

Gene	Primers	PCR Regimen	Reference
MDR1	CCCATCATTGCAATAGCAGG GTTCAAACTTCTGCTCCTGA	1	(17)
MRP	ATGTCACGTGGAATACCAGC GAAGACTGAACTCCCTTCCT	2	(18)
GST-π	CTCAAAGCCTCCTGCCTATA GTTGGTGTAGATGAGGGAGA	2	(18)
BUDP	GGTGTATCGATTGGTTTTTGC CATGGCGCCTTTGCTC	3	Selected by authors
Topo-IIα	GTAGCAATAATCTAAACCTCT GGTTGTAGAATTAAGAATAGC	2	(18)
Topo-IIβ	AAGCACTTTAGCAAGGCTAC CTACTGTGTTTCTGTCCACT	2	(18)
TK-1	TTCTCGGGCCGATGTTCTCA CAGAACTCCATGATGTAGG	2	(18)
HSP-70	GTCCTTACTATTGACGCAGG ATAGGCCACAGCCTCATCTG	4	(19)
c-fos	ATGTTCTCGGGCTTCAACGCAGACTAC GTACAGGAAGCCTCTAGGGAAGATGTG	5	(20)
c-jun	TCCTTAAGAACACAAAGCGG AAACAACACTGGGCAGGATA	6	(21)
bcl-2	GGATTGTGGCCTTCTTTGAG CCAAACTGAGCAGAGTCTTC	2	(18)
p53	GAAGACCCAGGTCCAGATGA GGTAGGTTTTCTGGGAAGGG	7	Selected by authors
β-actin	GACAACGGCTCCGGCATGTGCA TGAGGATGCCTCTCTTGCTCTG	1–7	(18)

Note: PCR Regimes: 1. 94°C/30 sec, 55°C/1 min, 72°C/2 min for 30 cycles. 2. 94°C/4 min, 55°C/1 min, 72°C/1 min for 1 cycle; 94°C/1 min, 55°C/50 sec, 72°C/1 min for 28 cycles, 60°C for 10 min. 3. 94°C/2 sec, 55°C/20 sec, 72°C/0 sec for 30 cycles. 4. 94°C/30 sec, 94°C/30 sec, 60°C/30 sec, 72°C/1 min for 38 cycles, 72°C/3 min. 5. 94°C/1 min, 50°C/2 min, 72°C/2 min for 40 cycles. 6. 94°C/30 sec, 55°C/75 sec, 72°C/15 sec for 27 cycles; 72°C/6 min. 7. 94°C/2 sec, 57°C/30 sec, 72°C/0 sec for 30 cycles.

The first method was based on the measurement of the enrichment of cell cytoplasmic fraction (lysate) and media by mono- and oligonucleosomes using a cell death detection ELISA kit (Boehringer). The measurement in the cell lysates was used as a characteristic of apoptosis, while the detection of nucleosomes in media reflected secondary necrosis, which accompanied apoptosis in *in vitro* experiments. The method was used to analyze time (0, 12, 24, 36, 48, 60, and 72 h) and concentration (10 different concentrations ranging from $0.1 \times IC_{50}$ to $50 \times IC_{50}$) dependent apoptosis induction in A2780 and A2780/AD cells.

The second method of apoptosis detection was based on the detection of single- and double-stranded DNA breaks (nicks) occurring at early stages of apoptosis by an *in situ* cell detection kit (Boehringer) using terminal deoxynucleotidyl transferase mediated dUTP-fluorescein nick end labeling method (TUNEL). Apoptotic cells were fixed and permeabilized according to the manufacturer's recommendations. Subsequently, the cells were incubated with the TUNEL reaction mixture. After washing, the label incorporated at the damaged sites of the DNA was visualized by fluorescence microscope (Nikon, Japan) and quantitated by flow cytometry (Ortho Cytofluorograph IIS). Cells were considered apoptotic when their fluorescence intensity was greater than the mean value + 3 standard deviations for cells stained with labeling solution in the absence of terminal deoxynucleotidyl transferase (negative control).

DNA Isolation

Genomic DNA was isolated using QIAamp blood and cell culture DNA isolation kits (Qiagen). The purity and concentration of DNA were simultaneously analyzed by 0.6% agarose gel electrophoresis and by spectrophotometric measurements. In the former case DNA concentration (per cell) was calculated from the AUC for the DNA band (after ethidium bromide staining) using a Low DNA Mass ladder (GibcoBRL) as a mass standard. During the spectrophotometric measurements, a molar extinction coefficient of 50 ng of DNA per unit of optical density at 260 nm was used. DNA was considered to be pure if both the ratio of optical density at 260/280 nm was greater than 1.8 and smaller than 2.0 relative units and the gel electrophoresis showed only one distinct DNA band.

Lipid Peroxidation

Lipid peroxidation in cell lysates (1×10^7 cells) was assessed by the measurement of lipid peroxides using the commonly accepted thiobarbituric acid (TBA) reaction (22). Our modification of the method included a 5–10 min incubation of 240 μl of cell lysate at 95°C with 12 μl of 2g/l of butylated hydroxytoluene (2,6-di-tert.-butyl-p-cresol), 120 μl of 100 g/l trichloroacetic acid, and 120 μl of 16 g/l TBA (all from Sigma). After cooling with tap water and centrifugation, 250 μl of the supernatant were transferred to a microtiter plate and the optical density was measured at 532 nm. The concentration of lipid peroxides was expressed in nmol of malondialdehyde using 1,1,3,3-tetrametoxypropane (Sigma) as an external standard.

Statistics

Data obtained were analyzed as described above using programs written by Dr. V. Pozharov. The difference between variants was considered significant if $P < 0.05$, determined by single factor analysis of variance (ANOVA).

RESULTS

Cytotoxicity

In the major part of the experimental work cells were incubated with 10 different concentrations of drugs in media

ranging from the 1 × IC_{50} dose to the 10 × IC_{50} dose (ADR accumulation) or from the 0.1 × IC_{50} to the 50 × IC_{50} (other dose-dependent studies). In some experiments only two concentrations were used, namely the 1 × IC_{50} and the 10 × IC_{50} doses. The IC_{50} doses, determined in the preliminary experiments (8), were 0.18 ± 0.01 and 6.92 ± 1.52 μM for free ADR, A2780 and A2780/AD cells respectively; 36.35 ± 0.52 and 43.31 ± 3.02 μM for P(GFLG)-ADR, A2780 and A2780/AD, respectively.

Data presented in Fig. 1 show that after the incubation of A2780 cells with the 1 × IC_{50} dose of free ADR almost all drug was accumulated in the cells or associated with the cell surface, while its concentration in media after the incubation was less than the sensitivity threshold of the method used. At high doses of ADR about 80% of the drug was associated with the cells. In contrast, the incubation of A2780/AD resistant cells led to the accumulation of only about 15–20% of ADR. In both cases the relation between ADR concentrations in the media and in cell lysates was close to linear (Fig. 1 A, B). In the case of P(GFLG)-ADR a more significant concentration gradient between cells and media was detected and the relationship was substantially non-linear (Fig. 1 C, D). Moreover, it appeared that P(GFLG)-ADR accumulated slightly more in the A2780/AD resistant cells than in A2780 sensitive ones (Fig. 1 C, D).

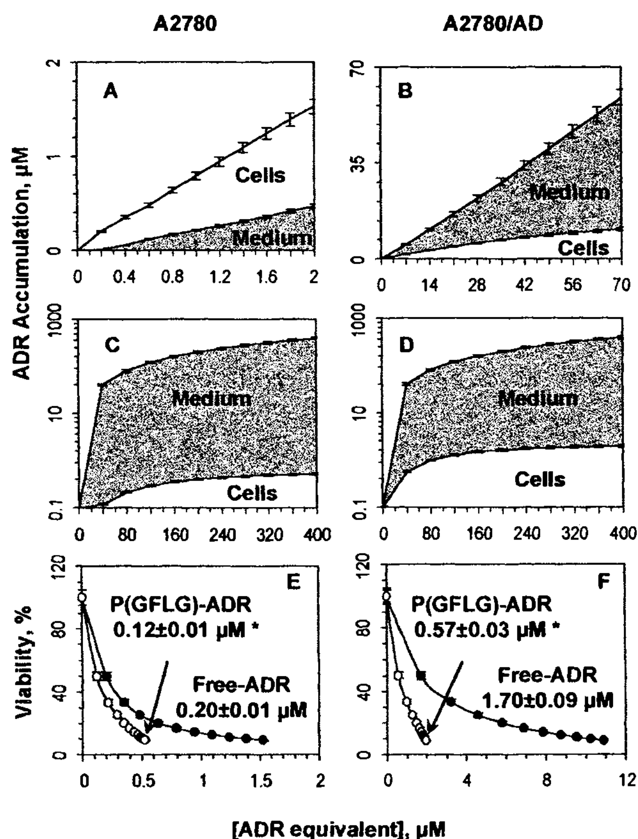


Fig. 1. ADR accumulation (A-D) and cell viability (E, F) in A2780 sensitive and A2780/AD ADR-resistant human ovarian carcinoma cells. ADR accumulation was measured by HPLC, cell viability by MTT assay as described in the methods. Abscissa represents ADR concentrations in media (A-D) or cell lysates (E, F). Means ± SD from 4 independent measurements are shown. E, F, closed symbols—free ADR, open symbols—P(GFLG)-ADR.

Due to the different mechanisms of cell entry of free and HPMA copolymer-bound ADR, we analyzed their cytotoxicity based on intracellular drug concentrations. The analysis of cell viability vs. intracellular ADR concentration (Fig. 1 E, F) suggested that the IC_{50} dose for P(GFLG)-ADR in A2780/AD cells was about three times less than the IC_{50} dose for free ADR.

To analyze the mechanisms of cellular toxicity and determine differences in the anticancer action of free ADR and P(GFLG)-ADR we studied the impact of both drugs on cell death induction, the influence on cell metabolism and cell signaling pathways, as well as on the mechanisms which protect cells from the damaging action of xenobiotics.

Apoptosis

DNA damage and apoptosis are the main mechanisms of action of many anticancer drugs including ADR (23). We used two methods to measure the fraction of apoptotic cells-TUNEL and detection of nucleosomes in the cytoplasm and media by ELISA.

Typical fluorescence microscope images obtained after incubation of A2780 and A2780/AD cells with free ADR and P(GFLG)-ADR using the TUNEL technique are shown on Fig. 2. It appeared that the incubation of cells with P(GFLG)-ADR induced apoptosis more significantly than the incubation with free ADR. Analysis of TUNEL labeled cells by flow cytometry revealed that about 4% of control A2780 and A2780/AD cells underwent apoptosis. At the 1 × IC_{50} dose, P(GFLG)-ADR was about 30% more effective in A2780/AD resistant cells (30.9 ± 1.1% of apoptotic cells vs. 23.2 ± 1.2%, $P < 0.05$, after incubation with P(GFLG)-ADR and free ADR, respectively), and up to two times more effective in A2780 sensitive cells (46.9 ± 2.1% of vs. 25.5 ± 1.2%, $P < 0.05$) than free ADR. At a 10 × IC_{50} dose, the apoptosis induction with P(GFLG)-ADR was substantially more pronounced than with free ADR: almost two times in sensitive cells (63.7 ± 3.0% vs. 34.8 ± 1.7%, $P < 0.05$), and more than ten times in resistant cells (48.1 ± 2.3% vs. 3.6 ± 0.1%, $P < 0.05$).

To further characterize apoptotic DNA cleavage, mono- and oligonucleosomes in the cell lysates (an indication of apoptosis) and in the media (an indication of secondary necrosis following in vitro apoptosis) were detected. An increase in cell-associated ADR concentration lead to a progressive increase in apoptosis. The latter was accompanied by secondary necrosis when cells were exposed to HPMA copolymer-bound ADR (Fig. 3 A-D). In contrast, cells that survived the action of high doses of free ADR were more resistant; neither apoptosis nor necrosis were detected at these conditions.

Time-dependent induction of apoptosis was studied using two concentrations of the drugs, which corresponded to the 1 × IC_{50} dose and the 10 × IC_{50} dose (Fig. 4). The results demonstrated that when 1 × IC_{50} dose of drugs was used, an increase in the incubation time progressively increased the level of apoptosis, as detected by the increase of the nucleosome concentration in the cytoplasm (Fig. 3 A, B). The activation of apoptosis was more significant after incubation with P(GFLG)-ADR. In addition, both drugs stimulated cell destruction by secondary necrosis, which increased the accumulation of low mass DNA fragments in the media (Fig. 4 C, D). However,

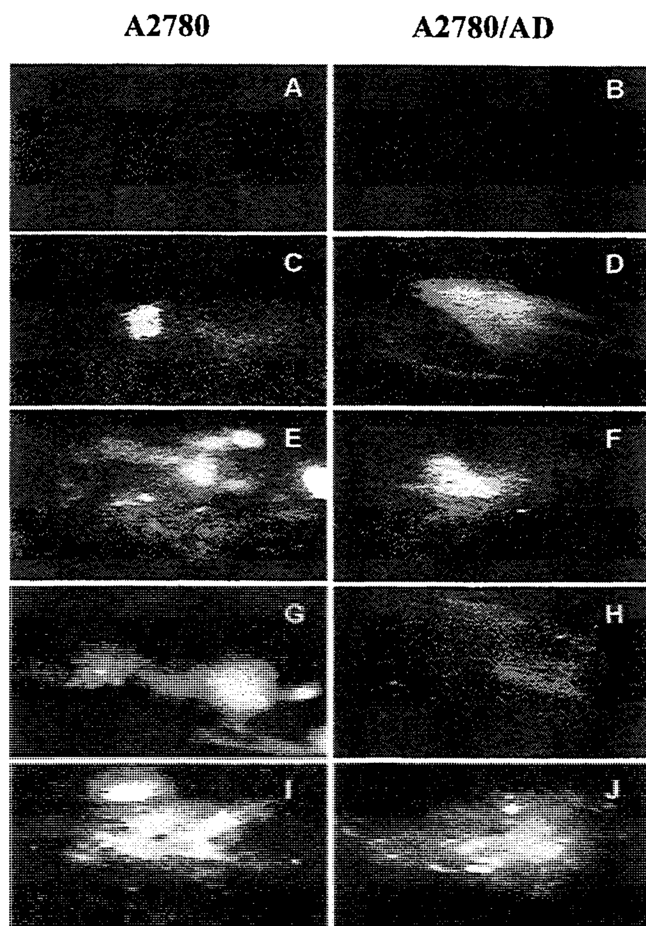


Fig. 2. Typical fluorescence microscopy images of TUNEL-labeled A2780 and A2780/AD human ovarian carcinoma cells (1×10^7) after exposure to free ADR (C, D, G, H) and P(GFLG)-ADR (E, F, I, J). A, B—Control; C-F—$1 \times IC_{50}$ dose; G-J—$10 \times IC_{50}$ dose.

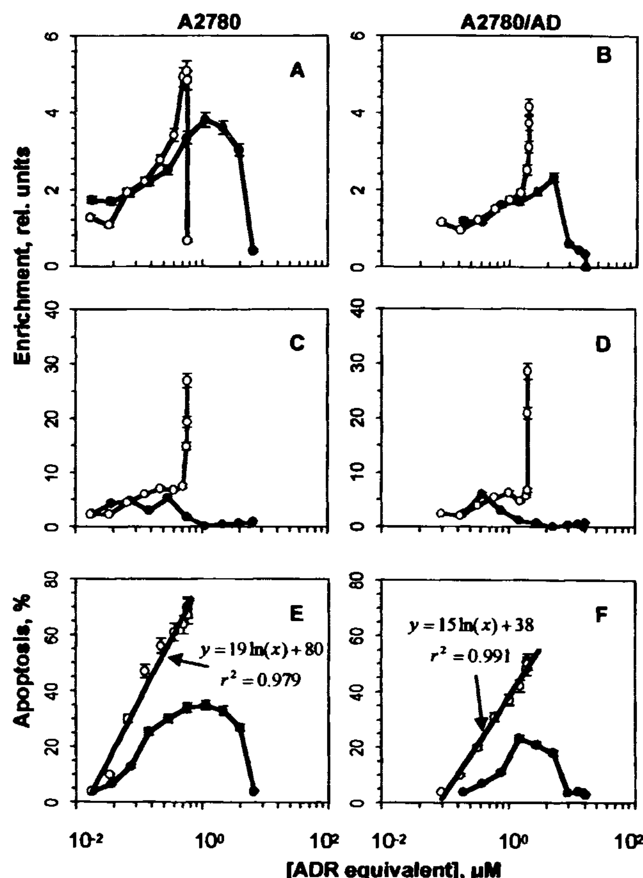


Fig. 3. Concentration-dependent apoptosis induction by free ADR (closed symbols) and P(GFLG)-ADR (open symbols) in A2780 sensitive and A2780/AD ADR-resistant human ovarian carcinoma cells. Enrichment of cell lysates (A, B) and media (C, D) by mono- and oligonucleosomes was measured by ELISA; percentage of apoptotic cells was determined by flow cytometry after TUNEL-labeling as described in the methods section (E, F). Abscissa represents the ADR concentration in cell lysates (A, B) or media (C, D), the ordinate the enrichment of nucleosomes (A-D) or percent of apoptotic cells (E, F). Means ± SD from 4 independent measurements are shown.

after a certain time period (60 h for A2780 sensitive and 24 h for A2780/AD resistant cells) the degree of apoptosis associated secondary necrosis produced by free ADR began to decrease. Similar results were obtained when the $10 \times IC_{50}$ dose was used (Fig. 4 E-H). In the latter case the activation of apoptosis was more pronounced, and differences between free and HPMA copolymer-bound ADR were more significant. In general, these experiments revealed that i) total apoptotic DNA fragmentation registered both in cell cytoplasm and media was much more significant when cells were incubated with P(GFLG)-ADR when compared to free ADR; and ii) incubation of cells with free ADR led, after a certain period of time, to increased cellular resistance against DNA damage produced by ADR. One possible mechanism of this phenomenon may be a different influence of free and HPMA copolymer-bound ADR on tissue metabolism.

Cellular Metabolism, DNA Content and Lipid Peroxidation

To estimate the influence of free and HPMA copolymer-bound ADR on cellular metabolism, the glucose consumption (which reflects total energy production), mitochondrial activity (which reflects aerobic metabolism) and lactate accumulation (which reflects anaerobic metabolism) were evaluated (Fig. 5). It was found that glucose consumption per cell increased with the increase of free ADR concentration. The same effect was found for aerobic energy exchange. The changes in anaerobic metabolism were less significant. It is very important to note that the HPMA copolymer-bound ADR did not demonstrate such an effect.

Energy exchange modifications have an impact on DNA synthesis. We found that free ADR significantly decreased the DNA concentration per cell only at very high concentrations and only in A2780 sensitive cells (Fig. 6 B). In contrast, HPMA copolymer-bound ADR inhibited DNA synthesis both in A2780 sensitive and A2780/AD resistant cells. This probably reflects the limitation of cell metabolism and the development of lactate-acidosis under the experimental conditions used. These data seem to be corroborated by the detection of DNA damage by the TUNEL method (Fig. 3).

Another factor contributing to the cytotoxicity of drugs similar to ADR is lipid peroxidation. Data presented on Fig. 6 A show that at low drug concentrations both free and HPMA

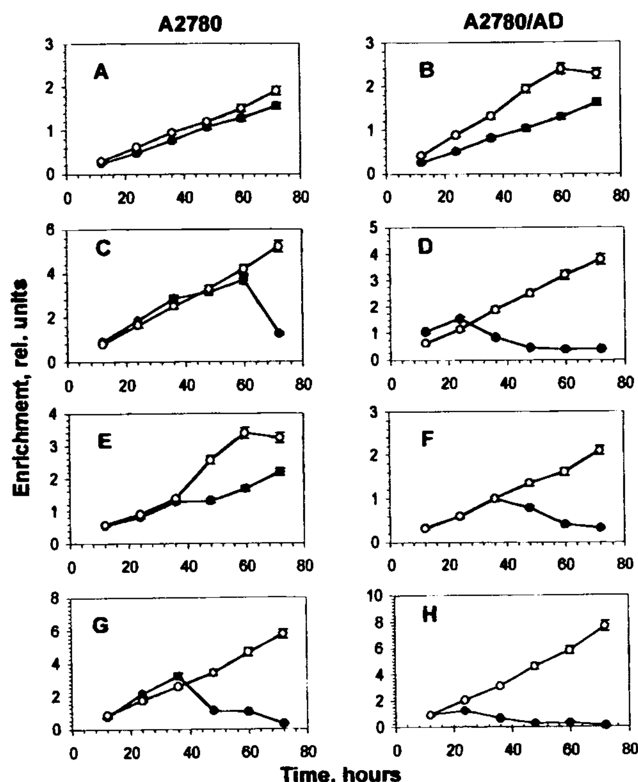


Fig. 4. Time-dependent apoptosis induction by free ADR (closed symbols) and P(GFLG)-ADR (open symbols) in A2780 sensitive and A2780/AD ADR-resistant human ovarian carcinoma cells. Enrichment of cell lysates (A, B, E, F) and media (C, D, G, H) by mono- and oligonucleosomes was measured by ELISA. Concentrations of drugs were equivalent to the $1 \times IC_{50}$ dose (A-D) and the $10 \times IC_{50}$ dose (E-H). Means ± SD from 4 independent measurements are shown.

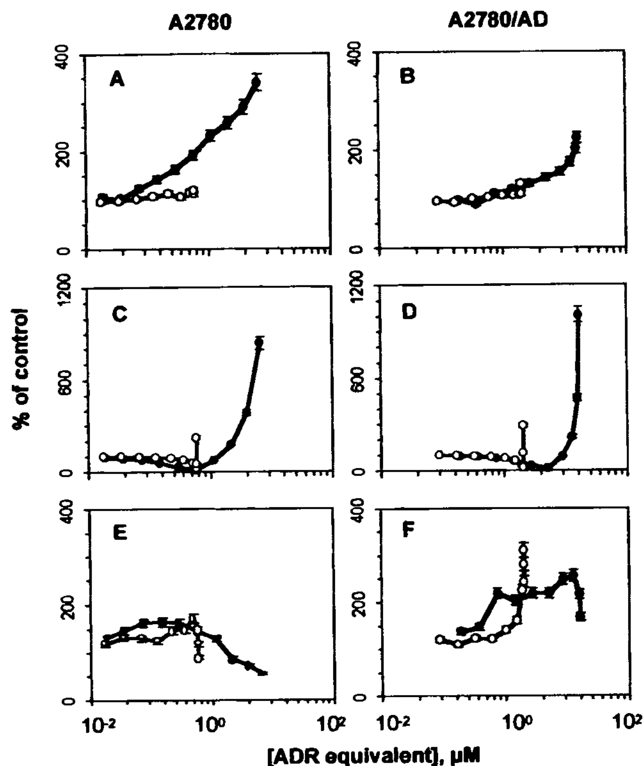


Fig. 5. Total glucose consumption (A, B), mitochondrial activity (C, D) and lactate accumulation (E, F) in A2780 sensitive and A2780/AD ADR-resistant human ovarian carcinoma cells after the incubation with free ADR (closed symbols) and P(GFLG)-ADR (open symbols). Glucose consumption and lactate concentration were measured using enzymatic assays, mitochondrial activity was estimated by the MTT assay as described in the methods section. Abscissa represents the ADR concentration in cell lysates. A2780 and A2780/AD cells incubated with media without drug were used as controls. Means ± SD from 4 independent measurements are shown.

copolymer-bound ADR significantly induced lipid peroxidation. On the other hand, only HPMA copolymer-bound ADR was able to activate lipid peroxidation at high drug concentrations.

Gene Expression

To further understand the mechanisms of biological action of free and HPMA copolymer-bound ADR we analyzed their influence on the expression of certain genes in A2780 sensitive and A2780/AD resistant cells. Pathways known to be associated with drug resistance (18,24–28) were chosen for the screening: a) *MDR1* and *MRP* genes encoding transmembrane efflux pumps; b) *GST-π* and *BUDP* genes, encoding glutathione and UDP transferases active in the detoxification of xenobiotics; c) *Topo-II-α*, *Topo-II-β*, and *TK-1* genes encoding topoisomerases and thymidine kinase responsible for DNA repair, replication and biosynthesis of nucleotides; d) *HSP-70* gene encoding a heat-shock protein related to non-specific cell resistance; e) *p53*, *c-fos*, *c-jun* and *bcl-2* genes involved in the regulation of apoptosis.

The expression of these genes was studied by quantitative RT-PCR using *β-actin* as an internal standard. The results are shown in Fig. 7. The results are clearly complex and a detailed explanation of the differences in cell signaling pathways after exposure of cells to free and HPMA copolymer-bound ADR will require additional studies. Nevertheless, the data point out the main differences in biological activities of free ADR and P(GFLG)-ADR on a subcellular level.

It should be stressed that in most cases the influence of both drugs on gene expression was time and concentration dependent. Regardless of the direction of change in the expression (up- or down-regulation) the degree of change increased with the time of incubation and drug concentration. This tendency was almost the sole feature common for free and HPMA copolymer-bound ADR. All other characteristics of action on gene expression, including the level and sometimes even the direction of changes, were different.

Incubation of A2780 sensitive cells with high concentrations of free ADR induced the expression of the *MDR1* gene after 72 h, whereas HPMA copolymer-bound ADR did not demonstrate such an effect. In addition, P(GFLG)-ADR significantly down-regulated (to undetectable levels) the expression of the *MRP* gene encoding the multidrug resistance associated protein ATP driven non-P-glycoprotein efflux pump, while free ADR was not able to suppress the *MRP* gene expression.

Another difference between free and HPMA copolymer-bound ADR was found in their impact on the detoxification activity. The $1 \times IC_{50}$ dose of free ADR did not significantly change the expression of the *GST-π* gene and substantially

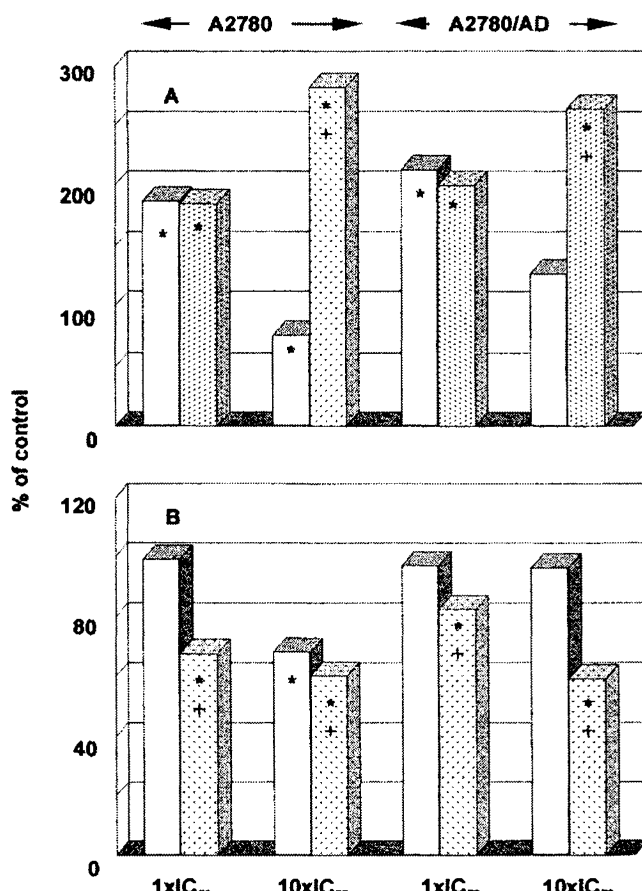


Fig. 6. Lipid peroxidation (A) and DNA content per cell (B) in A2780 sensitive and A2780/AD ADR resistant human ovarian carcinoma cells after incubation with free ADR (open bars) and P(GFLG)-ADR (patterned bars). Activity of lipid peroxidation was estimated by the accumulation of TBA reactive substances in cell lysates; DNA was isolated and assayed as described in the methods section. A2780 and A2780/AD cells incubated with media without drug were used as controls. Means ± SD from 4 independent measurements are shown. * $P < 0.05$ when compared to control. $^{+}$ $P < 0.05$ when compared to free ADR.

activated the *BUDP* gene in A2780 sensitive cells. At the $10 \times IC_{50}$ dose, both genes were over-expressed and the degree of expression was time-dependent—the longer the incubation time, the higher the activation. A similar situation was observed in A2780/AD resistant cells at both concentrations of free ADR. In contrast, P(GFLG)-ADR inhibited the expression of these genes often to non-detectable levels.

The influence of the two forms of ADR on the topoisomerase and thymidine kinase activities was also different. While free ADR up-regulated the *Topo-IIα* gene in A2780 sensitive cells, HPMA copolymer-bound ADR down-regulated it to undetectable levels. Both free and HPMA copolymer-bound ADR decreased the expression of the *Topo-IIα* gene in A2780/AD cells. However, the degree of the reduction was more significant when cells were incubated with P(GFLG)-ADR. Free ADR produced opposite effects on *Topo-IIβ* gene expression at low and high concentrations. At relatively low concentrations no effect was detected in A2780 sensitive cells, whereas the up-regulation of the *Topo-IIβ* gene was found in the A2780/AD resistant cells. At high concentrations free ADR significantly inhibited the expression of the *Topo-IIβ* gene in both sensitive and resistant cells. In contrast, HPMA copolymer-bound ADR down-regulated the *Topo-IIβ* gene at low and high concentrations in both types of cells. *TK-1* gene was up-regulated by low concentrations of free ADR in A2780 sensitive and A2780/AD resistant cells. High concentrations of free ADR led to the up-regulation in A2780 sensitive cells and down-regulation in A2780/AD resistant cells of the *TK-1* gene. On the other hand, the action of P(GFLG)-ADR on the *TK-1* gene was similar to the influence on topoisomerases—down-regulation to non-detectable levels.

The expression of the gene encoding the adaptive *HSP-70* protein was not detected in A2780/AD resistant cells. In sensitive A2780 cells the gene was activated at low concentration and down-regulated at high concentrations of free ADR. Both low and high concentrations of HPMA copolymer-bound ADR down-regulated the *HSP-70* gene. Again, the degree of down-regulation was substantially more pronounced (to non-detectable levels) in case of P(GFLG)-ADR.

Measurements of the expression of the genes involved in the signaling pathways of apoptosis revealed the following. While the *c-fos* gene was overexpressed in A2780 sensitive cells at high doses and/or longer incubation periods of both drugs, it was significantly down-regulated in A2780/AD cells at similar conditions. The effect of both drugs on the *c-jun* gene expression was opposite—except for low concentration of free ADR in sensitive cells, it was down-regulated in sensitive cells and up-regulated in resistant cells. On the other hand, the expression of the *bcl-2* gene was increased by free ADR and was down-regulated by P(GFLG)-ADR in both A2780 and A2780/AD cells. Exposure to free and HPMA copolymer-bound ADR led to the up-regulation of the *p53* gene. The expression was more pronounced when the cells were exposed to P(GFLG)-ADR.

DISCUSSION

The measurement of cell viability as a function of drug concentration in the cell-surrounding media reflects two different processes—drug internalization by cells and the actual cytotoxicity of ADR inside and at the surface of cells. To analyze these processes separately, we have, in addition to monitoring the concentration of drugs in the medium, measured the amount of drugs associated with the cells. The data obtained show that after incubation of A2780 cells with free ADR about 80% of the drug was associated with the cells (surface and intracellular). In contrast, the incubation of A2780/AD resistant cells with free ADR resulted in a substantial decrease of ADR accumulation (Fig. 1). These findings indicate the presence of efflux pumps in the A2780/AD resistant cells responsible for the decrease of intracellular ADR concentration. However, no impact of the efflux pumps on the internalization of HPMA copolymer-bound ADR was detected. In fact, it appeared that the A2780/AD cells that over-expressed the *MDR1* gene encoding P-gp seemed to accumulate P(GFLG)-ADR even faster than A2780 cells. This suggests a probable increase in endocytotic activity of the resistant cells. The results also corroborated our previously published data (8,9) showing that HPMA copolymer-bound ADR overcame the *MDR1* and *MRP* gene-encoded multidrug resistance.

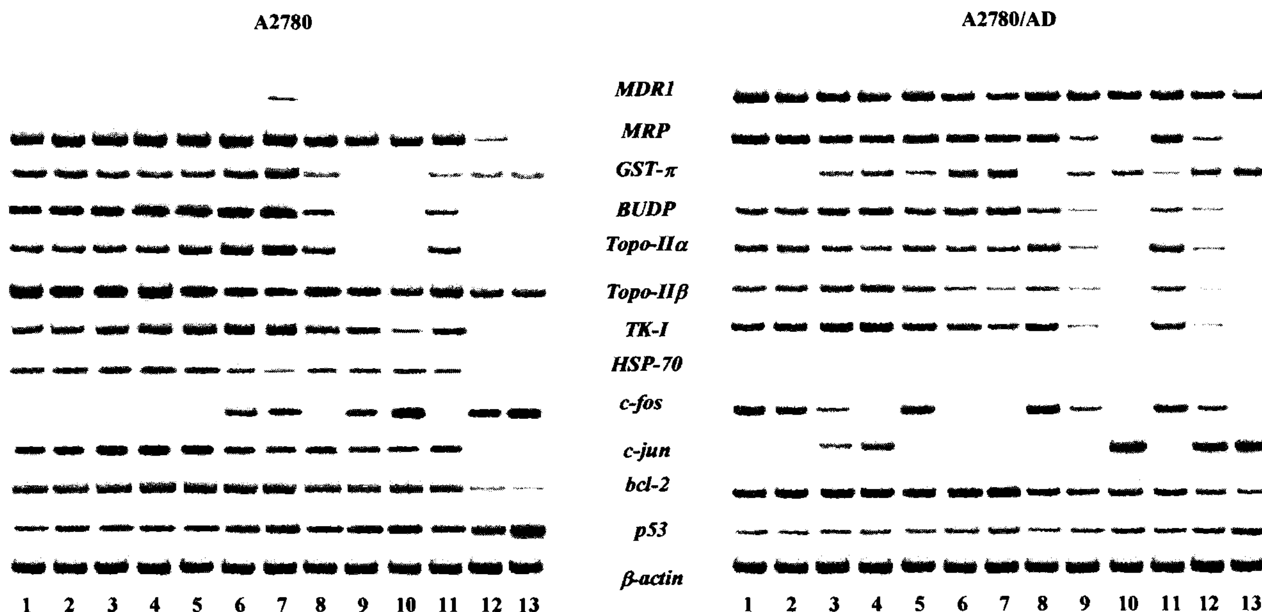


Fig. 7. Typical images of gel electrophoresis of RT-PCR products from A2780 sensitive (left panel) and A2780/AD ADR-resistant (right panel) human ovarian carcinoma cells. Identical numbers of cells, amounts of RT products, and volumes of PCR products were used for each analysis. See Table 1 for primers and PCR regimes used for individual genes. RT-PCR products from cells indicated below were loaded and electrophoresed in the 0.6–4% MetaPhor agarose gel: Lane 1, control cells; Lane 2, cells after 24 h incubation with an $1 \times IC_{50}$ dose of free ADR; Lane 3, cells after 48 h incubation with an $1 \times IC_{50}$ dose of free ADR; Lane 4, cells after 72 h incubation with an $1 \times IC_{50}$ dose of free ADR; Lane 5, cells after 24 h incubation with a $10 \times IC_{50}$ dose of free ADR; Lane 6, cells after 48 h incubation with a $10 \times IC_{50}$ dose of free ADR; Lane 7, cells after 72 h incubation with a $10 \times IC_{50}$ dose of free ADR; Lane 8, cells after 24 h incubation with an $1 \times IC_{50}$ dose of P(GFLG)-ADR; Lane 9, cells after 48 h incubation with an $1 \times IC_{50}$ dose of P(GFLG)-ADR; Lane 10, cells after 72 h incubation with an $1 \times IC_{50}$ dose of P(GFLG)-ADR; Lane 11, cells after 24 h incubation with a $10 \times IC_{50}$ dose of P(GFLG)-ADR; Lane 12, cells after 48 h incubation with a $10 \times IC_{50}$ dose of P(GFLG)-ADR; Lane 13, cells after 72 h incubation with a $10 \times IC_{50}$ dose of P(GFLG)-ADR.

It should be mentioned that the determination of ADR in cell lysates does not account for the surface drug concentration. It is known that ADR possesses surface activity (29). In addition, a certain amount of intracellular drug might be released into the medium after primary or secondary necrosis. However, data on the apoptotic activation seem to suggest that at lower drug concentrations the latter contribution should be minor. In spite of the shortcomings of the method used, it appears to be safe to conclude that P(GFLG)-ADR possesses a similar order of magnitude of cytotoxicity as free ADR, when the comparison is made based on intracellular (cell-associated) concentrations.

This finding indicates that in addition to the ability to overcome an action of transmembrane efflux pumps, HPMA copolymer-bound ADR has different mechanisms of biological action. The differences between P(GFLG)-ADR and free ADR are more pronounced in A2780/AD resistant cells.

The main mechanism of cell death by the action of the majority of anticancer drugs is an induction of apoptosis (23). Analysis of data obtained using TUNEL labeling of apoptotic cells and measurement of nucleosome accumulation in the cells and media revealed substantial differences in death induction by free and HPMA copolymer-bound ADR. It appeared that incubation of cells with P(GFLG)-ADR induced apoptosis more significantly than incubation with free ADR. A positive logarithmic dependence was found between the ADR equivalent concentration and the degree of apoptosis induced after incubation of both A2780 and A2780/AD cells with P(GFLG)-ADR (Fig. 3 E, F). On the other hand, an increase in the dose of free ADR resulted first in an increase of the degree of apoptosis, which was followed by a progressive decrease in the apoptotic cell content. These data indicate that cells, which survived after the action of high doses of free ADR, were more resistant than those incubated with low ADR concentrations. The percentage of cells, which underwent apoptosis in the former case, was less than in cells that survived the exposure to low ADR concentrations.

It is well known that cells need energy to maintain their homeostasis. An adequate energy exchange is extremely important in such a critical condition as drug exposure when cells need additional energy for drug efflux by ATP-dependent pumps and intracellular drug detoxification. The experimental data show a positive correlation between the activity of cellular metabolism and cellular resistance on one hand, and the activation of energy metabolism under the action of free ADR on the other (compare Figs. 3 and 5). The data do not indicate that ADR stimulates energy exchange. However, they suggest that cells, which survived the incubation with high doses of free ADR, possessed an increased metabolism.

In addition, the data analysis suggests a strong correlation between the activity of metabolism and apoptosis during exposure of A2780 sensitive cells to free ADR and P(GFLG)-ADR. It was found that cells with a high aerobic metabolism and a low anaerobic metabolism were more resistant to the action of both drugs. This relationship was very clear when cells were incubated with HPMA copolymer-bound ADR, and it was more complicated after incubation of cells with free ADR, probably

because of the mentioned process of cell selection. Indeed, a linear positive correlation was found between the percentage of apoptotic cells and lactate concentration for P(GFLG)-ADR in A2780/AD resistant cells (r = 0.973), while a strong negative correlation was registered between the activity of apoptosis and mitochondrial activity (r = −0.925 and −0.980 for sensitive and resistant cells, respectively). For free ADR a similar correlation was found, but the correlation coefficients were different for low ADR concentrations (whole cell population) and for high ADR concentrations (selected resistant cells). In all cases the values of r varied from 0.85 to 0.99. Cells with a high anaerobic and a low aerobic energy exchange were less resistant to ADR induced apoptosis. Generally, it may be concluded that the inhibition of aerobic metabolism and the compensative activation of anaerobic metabolism induces apoptosis. This may be due to acidosis resulting from lactate accumulation; it is known that acidosis is one of the major stimuli inducing apoptosis (30). In addition, an activation of lipid peroxidation, an increase in the DNA damage, and decrease in the DNA content were more significant after the action of free ADR.

All the above mentioned data suggest that HPMA copolymer-bound ADR appears to be more toxic inside cells than free ADR. In other words, the cells have a lower resistance to P(GFLG)-ADR. One may propose that P(GFLG)-ADR could have different mechanisms of action on the factors which determine cellular resistance when compared with free ADR. Therefore the next step was the analysis of the resistance during the action of free and HPMA copolymer-bound ADR.

One of the important mechanisms of cell resistance to anticancer drugs is the activity of ATP driven efflux pumps, which decrease the intracellular concentration of toxic substances. As mentioned above, free ADR was effectively removed from the cells by P-gp and MDR pumps; however, their expression in the resistant cells had minimal effects on the internalization of P(GFLG)-ADR. In addition, high concentration of free ADR ($10 \times IC_{50}$) and relatively long exposures (72 h) stimulated the expression of *MDR1* and *MRP* genes in sensitive A2780 cells, which encoded P-gp and MRP efflux pumps, respectively. In contrast, P(GFLG)-ADR did not demonstrate such an action and down-regulated these genes both in resistant and sensitive cells.

An increase in the resistance of A2780 sensitive cells was found during incubation with free ADR before an activation of the P-gp efflux pump. Moreover, A2780/AD resistant cells increased their resistance to ADR after the exposure to free ADR despite the decrease in the expression of *MDR1* and *MRP* genes. These data corroborate our previous study, which showed that during several weeks of incubation of sensitive cells with free ADR, multidrug resistance developed before detectable expression of MDR1 and *MRP* genes (9). This phenomenon suggests that, in addition to ATP driven drug efflux pumps, there may be other mechanisms of resistance to ADR; free ADR and P(GFLG)-ADR appear to influence those mechanisms differently.

While both forms of the drug activated the detoxification enzyme encoded by the *GST-π* gene in A2780/AD cells, the degree of the activation was significantly less for P(GFLG)-ADR. In addition, free ADR activated the UDP transferase encoded by the *BUDP* gene, while P(GFLG)-ADR suppressed the *BUDP* gene expression to non-detectable levels. Thus, P(GFLG)-ADR not only overcame and inhibited the active drug efflux pumps, but it was also less susceptible to enzymatic modifications during subcellular trafficking.

Another factor, which may contribute to the efficacy of HPMA copolymer-bound ADR, was the inhibition of enzymatic DNA damage repair and replication mechanisms. It was found that incubation of sensitive A2780 cells with free ADR resulted in an increased expression of genes encoding topoisomerase II isoforms and thymidine kinase, which are responsible for DNA repair and replication (18). In contrast, P(GFLG)-ADR significantly suppressed the expression of *Topo-IIα* and *Topo-IIβ* genes, often to non-detectable levels.

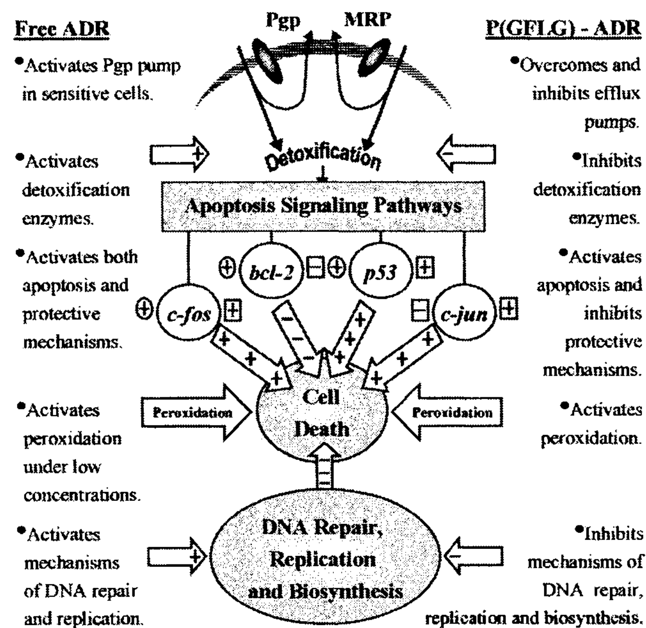
The biosynthesis of nucleotides and their homeostasis are related to the expression of thymidine kinase isoform 1 encoded by the *TK-1* gene. This gene was activated by free ADR in sensitive A2780 cells at all concentrations used; it was also activated in resistant A2780/AD cells when a low concentration of ADR was used. In contrast, P(GFLG)-ADR significantly decreased the expression of the *TK-1* gene in both cell lines for both concentrations.

Analysis of the expression of the *HSP-70* gene, which encodes the 70 kDa heat shock protein, indicated that the expression of this gene does not play a role in the ADR resistance of A2780/AD cells. However, an expression of the *HSP-70* gene was found in sensitive A2780 cells. Low concentrations of free ADR increased the expression of the *HSP-70* gene, while low concentrations of P(GFLG)-ADR decreased its expression in A2780 cells. High doses of both free and HPMA copolymer-bound ADR decreased the *HSP-70* gene expression in A2780 cells. The P(GFLG)-ADR was again found to be the more effective suppressor, because it suppressed the mechanisms responsible for the inactivation of ADR inside cells and repair of the DNA damage.

All the above mentioned facts suggested the possibility that free ADR and P(GFLG)-ADR may activate different cell signaling pathways or at least that the degree of activation of similar pathways may be different. Although the exact mechanisms of the activation, as well as the transducers involved in the process are still unknown, we tried to examine the most important genes involved in the programmed cell death. It is generally accepted (18,23) that DNA damage up-regulates the *p53* gene and therefore activates the central death signal, which includes two general pathways controlled by *c-fos*, *c-jun* genes (activators of cell death) and *bcl-2* gene, which acts as an inhibitor of apoptosis. The results suggested that the activation of the cell death pathways was more pronounced when cell were exposed to P(GFLG)-ADR. In addition, while free ADR also activated the *bcl-2* gene encoded apoptosis defensive mechanism, HPMA copolymer-bound ADR down-regulated the expression of this gene. The fact that P(GFLG)-ADR was a more effective activator of these pathways than free ADR could explain in part its higher efficacy as an apoptosis-inducing agent.

The peculiarities in the mechanisms of anticancer action of free and HPMA copolymer-bound ADR are summarized in Scheme 2 and include the following:

1. A high dose of free ADR activated the *MDR*1 gene, encoding the P-gp pump, in A2780 sensitive cells. In contrast, HPMA copolymer-bound ADR overcame existing P-gp and MRP pumps in A2780/AD resistant cells and inhibited the expression of the *MRP* gene both in sensitive and resistant cells.



Schematic 2. Peculiarities of biological action of free and HPMA copolymer bound ADR in human ovarian carcinoma cell lines. For detailed explanation see text. + activation − inhibition. Symbols in squares are related to HPMA copolymer-bound ADR, those in circles to free ADR.

2. Free ADR activated cell detoxification mechanisms by increasing the expression of genes encoding glutathione and UDP transferases, while HPMA copolymer-bound ADR suppressed the expression of these genes.

3. Both free and conjugated ADR induced apoptosis. However, HPMA copolymer-bound ADR activated apoptosis signaling pathways and, in addition, down-regulated the expression of the *bcl-2* gene which may result in the inhibition of apoptosis. In contrast, free ADR activated only one signaling cascade and the *bcl-2* gene. Consequently, apoptosis was less common after incubation of cells with free ADR when compared to HPMA copolymer-bound ADR.

4. The differences between free ADR and P(GFLG)-ADR increased with the time of incubation and drug concentration.

5. While both free ADR and P(GFLG)-ADR induced lipid peroxidation, free ADR was effective only at relatively low concentrations. HPMA copolymer-bound ADR activated lipid peroxidation both at low and high concentrations.

6. HPMA copolymer-bound ADR inhibited mechanisms of DNA repair, replication and synthesis, while free ADR activated DNA repair. This suggests that HPMA copolymer-bound ADR produced more DNA damage, which plays a central role in the development of programmed cell death-apoptosis.

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