Combination Chemotherapy and Photodynamic Therapy with Fab’ Fragment Targeted HPMA Copolymer Conjugates in Human Ovarian Carcinoma Cells

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Received January 9, 2008; Revised Manuscript Received July 13, 2008; Accepted July 14, 2008

Abstract: The biological activities of sequential combinations of anticancer drugs, SOS thiophene (SOS) and mesochlorin e6 monoethylenediamine (Mce6), in the form of free drugs, nontargeted N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer—drug conjugates, P-GFLG-Mce6 and P-GFLG-SOS (P is the HPMA copolymer backbone and GFLG is the glycylphenylalanylleucylglycine spacer), and Fab’-targeted HPMA copolymer—drug conjugates, P-(GFLG-Mce6)-Fab’ and P-(GFLG-SOS)-Fab’ (Fab’ from OV-TL16 antibodies complementary to CD47), were evaluated against human ovarian carcinoma OVCAR-3 cells. Mce6, SOS, P-GFLG-Mce6, P-GFLG-SOS, P-(GFLG-Mce6)-Fab’, and P-(GFLG-SOS)-Fab’, when used as single agents or in binary combination, exhibited cytotoxic activities against OVCAR-3 cells, as determined using a modified MTT assay. The binding and internalization of P-(GFLG-Mce6)-Fab’ and P-(GFLG-SOS)-Fab’ by OVCAR-3 cells were visualized by confocal microscopy and flow cytometry. The results confirmed an enhanced biorecognition by OVCAR-3 cells of Fab’-targeted HPMA copolymer conjugates over nontargeted conjugates. The median-effect analysis and the determination of the combination index (CI) were used to describe the drug interaction and quantify the synergism, antagonism, or additivity in anticancer effects. The sequential combinations of SOS+Mce6 and P-GFLG-SOS+P-GFLG-Mce6 displayed very strong synergism to synergism in the entire range of cell inhibition levels (fA0.5 - 0.95). The P-(GFLG-SOS)-Fab’+P-(GFLG-Mce6)-Fab’ exhibited a strong synergism for fA values up to about 0.85, but showed synergistic effect and nearly additive effect at fA = 0.9 and 0.95, respectively. These observations support the continuation of in vivo investigations of these conjugates for the treatment of ovarian cancer.

Keywords: N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymer; Fab’ antibody fragment; 2,5-bis(5-hydroxymethyl-2-thienyl)furan; mesochlorin e6 monoethylenediamine; combination index; ovarian cancer

Introduction

Polymer—drug conjugates are established as one of the first-generation nanomedicines for the treatment of cancer. Their use facilitates the uptake and transport of therapeutic agents and creates a dose-differentiation between the treatment target and the rest of the body, due to the fact that macromolecules can accumulate passively in solid tumor tissue by the phenomenon called “enhanced permeability and retention (EPR) effect” (1). The enhanced drug accumulation in tumor tissue increases the...
Seymour, L. W.; Duncan, R.; Strohalm, J.; Kopecˇek, J. Effect of and/or acti
active uptake by fluid-phase pinocytosis (nontargeted polymer-
recognition.7,8 HPMA copolymers are one of the most fre-
target sites, and (d) a targeting moiety to mediate biomolecular
understanding that are stable in blood circulation and release the free drug at
target sites, and (d) a targeting moiety to mediate biomolecular

Moreover, the intracellular trafficking mechanism of polymer—
drug conjugates renders drug efflux pumps ineffective.5 To
obtain such multifunctional capabilities, polymer—drug conju-
gates have been designed to consist of (a) a nonimmunogenic

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To improve the therapeutic outcome and reduce the toxicity of anticancer agents, a novel concept of combining chemotherapy and photodynamic therapy (PDT), using HPMA copolymer bound drugs, was developed.\(^\text{27}\) The in vivo studies on two cancer models, Neuro 2A neuroblastoma induced in A/J mice\(^\text{28}\) and human ovarian carcinoma heterotransplanted in nude mice,\(^\text{17,29,30}\) demonstrated that combination therapy with HPMA copolymer-bound DOX (doxorubicin) and HPMA copolymer-bound Mce\(_6\) (meso- chlorin e\(_6\) monoethylenediamine) produced tumor cures which could not be obtained with either chemotherapy or PDT alone. Furthermore, significantly lower nonspecific toxicities were observed when compared to low molecular weight drugs. Previously, in vitro studies of the binary combination of free and HPMA copolymer-bound SOS [2,5-bis(5-hydroxymethyl-2-thienyl)furan, NSC 652287], DOX, and Mce\(_6\) in the treatment of human A498 renal carcinoma cells using the median-effect method showed that these combinations displayed synergistic-to-additive effects, depending on the cytotoxic mechanisms of each agent.\(^\text{31}\)

In the present study, Fab′-targeted and nontargeted HPMA copolymer–drug (SOS and Mce\(_6\)) conjugates for combination chemotherapy and PDT against human ovarian OVCAR-3 carcinoma cells were synthesized. SOS, a dithiophene compound, was used as the chemotherapy agent. Its mechanism of action consists of disrupting the p53-HDM-2 (human double minute-2) interaction, resulting in an increased p53 accumulation, thereby inducing cell cycle arrest and apoptosis.\(^\text{32–35}\) For PDT the second-generation synthetic photosensitizer, Mce\(_6\), was used. Photosensitizer molecules can be activated by specific wavelength of light and interact with molecular oxygen to produce reactive singlet oxygen, causing irreversible photodamage to cells resulting in cell death.\(^\text{36}\) The antibody Fab′ fragment was prepared from OV-TL16 antibody, which recognizes the OA-3 surface antigen, also known as CD47 or IAP (integrin-associated protein),\(^\text{37,38}\) overexpressed on most human ovarian carcinoma cells.\(^\text{39,40}\)

It was hypothesized that a combination of these agents may produce synergistic effects and has higher efficiency than each agent alone. Accordingly, the efficiency of free, nontargeted, and Fab′-fragment-targeted HPMA copolymer-bound SOS and Mce\(_6\) against OVCAR-3 cells as single agents and in combination was evaluated. The combination index (CI) analysis was used to quantify the synergism, antagonism, and additive effects of drug combinations.\(^\text{41–43}\)

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Materials and Methods

Materials. Mce6 was purchased from Porphyrin Products (Logan, UT). SOS was kindly supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Line. The human ovarian carcinoma cell line OVCAR-3 was purchased from American type Culture Collection. Cells were cultured in RPMI 1640 medium (Sigma) containing 10 µg/mL insulin (Sigma) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), at 37 °C in a humidified atmosphere of 5% CO2 (v/v).

Synthesis of Nontargeted HPMA Copolymer—SOS Conjugate (P-GFLG-SOS). P-GFLG-SOS (Scheme 1A) was synthesized by binding of SOS to the P-GFLG-ONp polymer precursor via an ester linkage.31 Briefly, P-GFLG-ONp was dissolved in DMF and mixed with DMF solution of SOS. 4-Dimethylaminopyridine (DMAP) was added, and the reaction was allowed to proceed for 72 h in the dark at room temperature. The product was isolated, after reduction of volume, by precipitation into a mixture of acetone:ether (3:1 (v/v)). The precipitate was dissolved in methanol and applied to a Sephadex LH-20 column with methanol as the mobile phase. The polymer band was collected, concentrated and reprecipitated. The product was a yellowish powder, with an yield of 61%.

Synthesis of HPMA Copolymer—Mce6 Conjugate Containing Maleimide Groups. P-(GFLG-Mce6)-MAL. This copolymer precursor was prepared in three steps. The reactions are shown in Scheme 2. First, a polymerizable derivative of Mce6, N-(methacryloylglycylphenylalaninylglycine Mce6 (MA-GFLG-Mce6), was synthesized by reacting MA-GFLG-ONp (60 mg, 0.103 mmol) with Mce6 (63.5 mg, 0.093 mmol) in DMF (~2 mL). The reaction solution was stirred at room temperature in the dark for 2 h. N,N'-disopropylethylamine (DIPEA; 18 µL, 0.103 mmol) was added and stirred continuing overnight. 1-Amino-2-propanol (~8 µL, 0.103 mmol) and a small amount of tert-octylpyrocatechol were added and DMF was removed under reduced pressure. The residue was isolated using a Sephadex LH-20 column with acetone:methanol:acetic acid (2:1:0.1) as the mobile phase. The fractions were collected and checked on TLC. The product fraction was evaporated to dryness, washed with ether, collected by filtration, and dried under vacuum. The molecular weight (Mw) of MA-GFLG-Mce6 was 1083.6 Da as determined by electrospray ionization mass spectrometry (ESI-MS). The product yield was 90 mg (73%). Second, the polymerizable precursor P-(GFLG-Mce6)-NH2 was prepared by radical copolymerization of HPMA (107.6 mg, 0.751 mmol), N-(3-aminopropyl)methacrylamide hydrochloride (APMA, 16.27 mg, 0.091 mmol; Polysciences, Warrington, PA), and MA-GFLG-Mce6 (76.9 mg, 0.068 mmol) in methanol (~1.8 mL) at 50 °C for 48 h, using 2,2'-azobisisobutyronitrile (AIBN; 19.27 mg) as the initiator. The polymerization mixture contained 12.5 wt% of monomers and 1.2 wt% of AIBN. The molar ratio of HPMA:APMA:

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Scheme 1. Chemical Structures of (A) Nontargeted and (B) Fab'-Targeted HPMA Copolymer–Mce₆ or –SOS Conjugates

Table 1. Characterization of Nontargeted HPMA Copolymer Conjugates, Polymeric Precursors, and Fluorescently Labeled HPMA Copolymer Conjugates

| structures                      | mol % of side chainsᵃ | mmol of ligand/g of polymer conjugate | no. of ligands/polymer chain (conjugate) | apparent Mₜ (kDa)ᵇ |
|---------------------------------|------------------------|--------------------------------------|----------------------------------------|---------------------|
| P-GFLG-Mce₆                    | 2.04                   | 0.125                                | 2.9                                    | 23                  |
| P-GFLG-SOS                     | 1.62                   | 0.106                                | 3.4                                    | 32                  |
| P-(GFLG-Mce₆)-NH₂              | 2.62                   | 0.153 (Mce₆)                         | 1.7 (Mce₆)                            | 11                  |
| P-(GFLG-SOS)-NH₂               | 8.11ᵇ                  | 0.472 (NH₂)                          |                                        |                     |
| P-(GFLG-Mce₆)-MAL              | 4.60                   | 0.270 (SOS)                          | 2.7 (SOS)                              | 10                  |
| P-(GFLG-SOS)-MAL               | 4.70ᵇ                  | 0.272 (NH₂)                          |                                        |                     |
| P-(GFLG-Mce₆)-MAL              | 6.02ᵈ                  | 0.142 (Mce₆)                         | 2.0 (Mce₆)                            | 14                  |
| P-(GFLG-SOS)-MAL               | 2.60                   | 0.329 (MAL)                          |                                        |                     |
| P-(GFLG-Mce₆)-FITC             | 4.14                   | 0.236 (SOS)                          | 5.2 (SOS)                              | 22                  |
| P-(GFLG-SOS)-FITC              | 3.36ᵈ                  | 0.192 (MAL)                          |                                        |                     |
| P-(GFLG-Mce₆)-(Fab'-FITC)       | f                      | 0.060ᵃ                               | 1.0 (FITC)                             | f                   |
| P-(GFLG-SOS)-(Fab'-FITC)        | f                      | 0.038ᵃ                               | 0.6 (FITC)                             | f                   |
| P-(GFLG-Mce₆)-(Fab'-FITC)ᵇ      | f                      | 0.063ᵃ                               | 3.1 (FITC per conjugate)               | f                   |
| P-(GFLG-SOS)-(Fab'-FITC)ᵇ      | f                      | 0.050ᵃ                               | 2.5 (FITC per conjugate)               | f                   |

ᵃ Determined by UV spectrophotometry in methanol: ε₃95 = 158 000 M⁻¹ cm⁻¹ for Mce₆, and ε₃95 = 33 000 M⁻¹ cm⁻¹ for SOS.ᵇ Apparent molecular weight (Mₜ) of polymers was estimated by size exclusion chromatography using AKTA/FPLC (Pharmacia) system equipped with a Superose 6 column, calibrated with polyHPMA fractions. PBS buffer pH 7.3 ± 30% (v) acetonitrile and 0.1 M acetate buffer pH 5.5 ± 30% (v) acetonitrile were used for polymer conjugates containing Mce₆ and polymer conjugates containing SOS, respectively.ᶜ Determined by ninhydrin assay.ᵈ Determined by 5-((2-(and-3)-S-(acetylmercapto)succinyl)amino)fluorescein assay (SAMSA assay, Molecular Probes).ᵉ Fluorescently labeled targeted conjugates were prepared by reacting P-(GFLG-Mce₆)-Fab' and P- (GFLG-SOS)-Fab' (Table 2) with 5-SFX.

MA-GFLG-Mce₆ was 82.5:10:7.5. The reaction mixture was purified on a Sephadex LH-20 column eluted with methanol. The polymer fraction was collected, and methanol was evaporated. The residue was precipitated in a mixture of acetone:ether (1:2 v/v). The precipitate was dissolved in water, dialyzed (mol wt cutoff 6–8 kDa) against DI water.
and lyophilized. The product yield was 65 mg (32%). Third, polymeric precursor P-(GFLG-Mce6)-MAL was prepared by reacting P-(GFLG-Mce6)-NH2 (55 mg, 0.026 mmol NH2 group) with succinimidyl trans-4-(maleimidomethyl)cyclohexane-1-carboxylate (SMCC; 17.36 mg, 0.052 mmol) (Soltec Ventures, Beverly, MA) and DIPEA (30 µL, 0.179 mmol) as the initiator. The molar ratio of NH2:SMCC:DIPEA was 1:2:3. The product was separated on a PD-10 column and eluted with PBS. Other samples (Mce 6 and SOS) to enhance the solubility of SOS.48

Preparation of Antibody Fab’ Fragment-Targeted HPMA Copolymer−Mce6/SOS Conjugates, P-(GFLG-Mce6)-Fab’ and P-(GFLG-SOS)-Fab’. The targeted conjugates were prepared by dissolving P-(GFLG-Mce6)-MAL or P-(GFLG-SOS)-MAL precursor in 20 mM MES buffer pH 6.5 and reacting with freshly prepared Fab’ fragment (polymer:Fab’ weight ratio = 1:2) overnight in the dark at 4 °C. The product was purified on a DEAE Sepharose Fast Flow ion exchange column (Pharmacia), eluted using 20 mM Bis-Tris buffer pH 6.5 with a gradient NaCl concentration of 0 to 0.5 M. The fraction corresponding to conjugate was confirmed by size exclusion chromatography using Superose 6 (HR 10/30) column. The structure and composition of polymer conjugates are summarized in Scheme 1B and Table 2.

Preparation of Fluorescein-Labeled Nontargeted HPMA Copolymer−Mce6/SOS Conjugates, P-(GFLG-Mce6)-FITC and P-(GFLG-SOS)-FITC. 6-(Fluorescein-5-carboxy-mido)hexanoic acid succinimidyl ester (5-SFX; ∼0.1 mg, 0.170 µmol) was dissolved in dimethylsulfoxide (25 µL) and DI water (100 µL). The polymer precursor P-(GFLG-Mce6)-NH2 or P-(GFLG-SOS)-NH2 (∼3 mg, ∼1 µmol of NH2) was dissolved in DI water (∼300 µL). The 5-SFX solution was added into the polymer solution. DIPEA (∼1 drop) was added into the reaction solution while stirring. The mixture was stirred at room temperature in the dark for 1 h. Saturated Na2HPO4 (∼20 µL) was added to stop the reaction. The product was separated on a PD-10 column and eluted with PBS.

Preparation of Fluorescein-Labeled Fab’-Targeted HPMA Copolymer−Mce6/SOS Conjugates, P-(GFLG-Mce6)-Fab’-FITC and P-(GFLG-SOS)-Fab’-FITC. P-(GFLG-Mce6)-Fab’ and P-(GFLG-SOS)-Fab’ were reacted with 5-SFX. The procedure was as described for P-(GFLG-Mce6)-FITC and P-(GFLG-SOS)-FITC. The characterization of fluorescently labeled copolymer conjugates is shown in Table 1.

Drug Stock Solution Preparations. SOS was dissolved in PBS containing cyclodextrin (5% (w/v) cyclodextrin in PBS/1 mg of SOS) to enhance the solubility of SOS.48 P-(GFLG-SOS)-NH2, P-(GFLG-Mce6)-Fab’, and P-(GFLG-SOS)-Fab’ were prepared in PBS. Other samples (Mce6 and P-GFLG-Mce6) were prepared in DI water. All stock

### Table 2. Characterization of Fab’-Targeted HPMA Copolymer−Mce6/SOS Immunocomjugates

| structures                              | wt % | molecular ratio | mol wt (kDa) |
|-----------------------------------------|------|-----------------|--------------|
| P-(GFLG-Mce6)-Fab’                     | 2:1.21:1.76:8 | 2:1:1 | 64           |
| P-(GFLG-SOS)-Fab’                      | 1:6:23:5:74:9 | 5:1:1 | 72           |

Note: Calculated from the composition of polymer (molecular ratio of drug, polymer, and Fab).
solutions were sterile-filtered. Drug contents were determined by UV spectrophotometry. All stock solutions were freshly prepared and gradually diluted with RPMI 1640 culture medium before use.

Confocal Microscopy. Fifty thousand OVCAR-3 cells were subcultured into an eight-chamber slide and incubated for 2 days at 37 °C in a humidified atmosphere of 5% CO2. The cells in each chamber were exposed to fluorescein-labeled copolymer conjugates (at 20 µM FITC equivalent) at 37 °C for 1 h in the dark. Cells were fixed with 2% paraformaldehyde for 20 min at room temperature and washed twice with PBS. The chamber slide was covered with a cover slide utilizing antifade reagent (Molecular Probes). The cell internalization of fluorescently labeled HPMA copolymer conjugates was imaged using a Zeiss (Thornwood, NY) LSM 510 confocal imaging system.

Flow Cytometry. OVCAR-3 cells (75,000 cells/well) were seeded into a 24-well plate and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO2. Cells in each well were exposed to the fluorescein-labeled copolymer conjugates (at 20 µM FITC equivalent) at 37 °C for 1 h in the dark. The medium was removed. The cell monolayer was rinsed twice with ice-cold PBS and detached from the well surface by incubation with TrypLE Express (Gibco) for 2 min. All steps were carried out on ice to minimize efflux of the sample. The cells were suspended with ice-cold PBS containing 0.2% FBS, maintained in suspension on ice in the dark and processed for flow cytometry utilizing FACScan.
Synergism of SOS and Mce6 against OVCAR-3 Cells

Scheme 3. Experimental Protocols Used in the Cytotoxicity Study

A Cells treated with single agents

- SOS or SOS conjugates
- Mce6 or Mce6 conjug.

Irradiation for 30 min

B Cells treated with sequential combinations

- SOS and SOS conjugates
- Mce6 or Mce6 conjug.

Irradiation for 30 min

Determination of Drug Interaction and Combination Index. In combination treatment studies, OVCAR-3 cells were treated with a dose range of SOS for 4 days followed by a dose range of Mce6 for 1 day, and irradiated for 30 min (n = 6 in single experiment), as shown in Scheme 3. After each step the drug was removed and the cells were washed with warm PBS. Drug interaction and CI were determined using median-effect analysis according to the method of Chou and Talalay.41,42 The median-effect equation describes dose–effect relationships, which is expressed by:

\[ f/D_m = (D/D_m)^a \]

or

\[ \log(f/D_m) = m \log(D) - m \log(D_m) \]

where

- \( f \) and \( D \) are the fraction affected [1 – (absorbance of treatment well – average of absorbance of blanks)/average of absorbance of untreated cell wells – average of absorbance of blanks)] and unaffected (\( f = 1 - f_a \)) by the dose or concentration \( D, D_m \) is the median-effect dose (IC50) that inhibits the cell growth by 50%, and \( m \) is the coefficient signifying the shape of the dose–effect relationship. Based on the logarithmic conversion, the plot of \( x = \log(D) \) versus \( y = \log(f/D_m) \) is called the median-effect plot and \( D_m \) is calculated from the antilog of the x-intercept. The CI describes the interaction between two drugs and quantitates the synergism, antagonism or additive effects. The CI is determined by the equation:

\[ CI = [(D_1)/(D_{1,0})] + [(D_2)/(D_{2,0})] \]

where \( D_1 \) and \( D_2 \) are the doses of drug 1 alone and drug 2 alone that inhibit the cell growth x%, respectively. \( D_{1,0} \) and \( D_{2,0} \) are for doses in combination that also inhibit x%. The CI values were calculated for different values of \( f_a \) and plotting the CI values as a function of \( f_a \) values, using CompuSyn software (CompuSyn Inc., Paramus, NJ). In the \( f_a–CI \) plot, CI < 1, = 1, and > 1 indicate synergism, additivity, and antagonism, respectively.

Statistical Analysis. All mean values are presented as means ± standard deviation (n = 6 in a single experiment).

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Results and Discussion

Characteristics of HPMA Copolymer–Mce6–SOS Conjugates. The structures of HPMA copolymer conjugates, P-GFLG-Mce6, P-GFLG-SOS, P-(GFLG-Mce6)-Fab, and P-(GFLG-SOS)-Fab, are shown in Scheme 1. The drugs, Mce6 and SOS, were bound to the HPMA copolymer backbone via a GFLG spacer, stable in the bloodstream, but susceptible to enzymatically catalyzed hydrolysis in the lysosomal compartment of the cells. For Fab' attachment, the amino groups of APMA monomer units in HPMA copolymer precursors were first converted to maleimido groups by reaction with a heterobifunctional agent, SMCC (Scheme 2), followed by attachment of Fab' via thioether bonds. For some experiments, fluorescently labeled conjugates were synthesized. In nontargeted conjugates, the 5-SFX was attached to amino groups of APMA monomer units. The Fab'-targeted conjugates were labeled by the reaction of 5-SFX with the final conjugates.

The characteristics of HPMA copolymer precursors, nontargeted HPMA copolymer conjugates, Fab'-targeted HPMA copolymer conjugates, and fluorescently labeled conjugates are summarized in Tables 1 and 2. P-GFLG-Mce6, P-GFLG-SOS, P-(GFLG-Mce6)-Fab', and P-(GFLG-SOS)-Fab' conjugates contained 2.9, 3.4, 2.0, and 5.0 drug molecules per macroolecule, respectively. P-(GFLG-Mce6)-Fab' and P-(GFLG-SOS)-Fab' had drug:polymer:Fab' molecular ratios of approximately 2:1:1 and 5:1:1, respectively. The $M_w$ of Fab'-targeted copolymer conjugates were 2.2 to 2.8 times higher than those of nontargeted conjugates.

Intracellular Uptake of Fluorescein-Labeled Fab'-Targeted HPMA Copolymer Conjugates. Unlike low molecular weight drugs that enter cells by diffusion through the plasma membrane, macromolecules are internalized within membrane-limited vesicles in the process of endocytosis. Several basic internalization mechanisms, clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin- and caveolin-independent endocytosis, and macropinocytosis have been identified. To a greater or lesser extent, two or more distinct mechanisms coexist when a single cell type internalizes macromolecule–drug conjugates.

Studies on the subcellular fate of HPMA copolymer–drug conjugates demonstrated that the conjugates are lysosomotropic and will accumulate in the lysosomal compartment of the cell. Tijerina et al., using subcellular fractionation, determined the localization of a considerable fraction of HPMA copolymer–Mce6 conjugates in the lysosomal compartment of human ovarian carcinoma A2780 cells. Ome-lyanenko et al. used pH dependent fluorescence of FITC to display the lysosomotropism of FITC-labeled HPMA copolymers containing N-acetylated galactosamine in HepG2 hepatocarcinoma cells.

Recently, the uptake mechanism of HPMA copolymer–DOX conjugate in human ovarian carcinoma OVCAR-3 cells was studied by confocal fluorescence microscopy and by colocalization experiments with substrates specific for a particular internalization mechanism. The results suggested that the HPMA copolymer–DOX conjugate is internalized via both clathrin- and caveola-mediated endocytosis.

The biorecognition and cellular uptake of P-(GFLG-Mce6)-(Fab'-FITC) and P-(GFLG-SOS)-(Fab'-FITC) was studied using confocal microscopy (Figure 1) and flow cytometry (Figures 2 and 3). Nontargeted conjugates, P-(GFLG-Mce6)-FITC and P-(GFLG-SOS)-FITC, served as controls. After a 1 h exposure of OVCAR-3 cells to HPMA copolymer conjugates at 37 °C, the intracellular concentrations of targeted polymer conjugates containing Mce6 and SOS were significantly higher when compared to nontargeted conjugates. Both confocal microscopy images and flow cytometry profiles displayed very similar results.

However, at 37 °C two processes, biorecognition at surface and internalization by endocytosis, are operative. To clearly demonstrate the biorecognition of targeted conjugates by the CD47 antigen, OVCAR-3 cells were exposed to conjugates at 0 °C. Flow cytometry data at 0 °C, at conditions that suppress endocytosis, demonstrated the biorecognition of P-(GFLG-Mce6)-Fab' and P-(GFLG-SOS)-Fab' conjugates by OVCAR-3 cells (Figure 3). After incubation of OVCAR-3 cells with targeted and nontargeted conjugates for 2 h, no increase in fluorescent intensity (when compared to controls not exposed to any conjugates) in cells incubated with P-GFLG-Mce6 and P-GFLG-SOS was observed. In contrast, higher fluorescent intensities were detected in cells incubated with targeted (Fab' containing) conjugates P-(GFLG-Mce6)-Fab' and P-(GFLG-SOS)-Fab'. Presumably, the fluorescent signals were derived from membrane associated conjugates as a result of Fab'–CD47 interactions.

These results indicated the biorecognition of HPMA copolymer conjugates containing the Fab' antibody fragment by OVCAR-3 cells. The results of confocal microscopy are
consistent with the internalization of targeted HPMA co-polymer conjugates via receptor-mediated endocytosis, and of the nontargeted conjugates, containing hydrophobic drugs, by fluid-phase pinocytosis and adsorptive pinocytosis, concurrently.

The data are consistent with our previous results on the determination of binding constants of OV-TL16 antibody targeted HPMA copolymers toward OVCAR-3 cells. The affinity constant, $K_a$, of free antibody was $8 \times 10^{-8}$ M$^{-1}$, whereas the $K_a$ for the P-(GG-Mce6)-Fab$'$ was $3 \times 10^{-8}$ M$^{-1}$. The minor decrease in the affinity may be a result of chemical modification and/or steric hindrance of the polymer chain upon the formation of the antibody—antigen complex.

**In Vitro Inhibition of OVCAR-3 Cell Growth by Drugs as Single Agents.** The growth inhibitory effects of Mce6, SOS, P-GFLG-Mce6, P-GFLG-SOS, P-(GFLG-Mce6)-Fab$'$, and P-(GFLG-SOS)-Fab$'$ as single agents on OVCAR-3 cells were evaluated after drug exposure using the MTT assay. The IC$_{50}$ values for the free drugs, nontargeted and targeted HPMA copolymer conjugates are shown in Table 3. The 1-day Mce6 exposure and 4-day SOS exposure have been selected based on preliminary experiments on the relationship between exposure time and cell inhibition effect (data not shown). After 1-day Mce6 exposure and 4-day SOS exposure, the cells were more susceptible to Mce6 than SOS about 2 times. It is...
Figure 3. Flow cytometry profiles of OVCAR-3 cells incubated with fluorescein-labeled HPMA copolymer conjugates in RPMI 1640 culture medium for 2 h in the dark at 0 °C. (A) Control cells, (B) P-(GFLG-Mce6)-FITC, (C) P-(GFLG-SOS)-FITC, (D) P-(GFLG-SOS)-(Fab′-FITC), (E) P-(GFLG-Mce6)-(Fab′-FITC).

Table 3. IC50 Values for Mce6, SOS, and HPMA Copolymer–Mce6−/−SOS Conjugates against OVCAR-3 Cells

| Drug          | Incubation time (days) | IC50 or Dm (µM) |
|---------------|------------------------|-----------------|
| Mce6          | 1                      | 3.34 ± 0.43     |
| P-GFLG-Mce6   | 1                      | 12.5 ± 1.06     |
| P-(GFLG-Mce6)−Fab′ | 1                    | 1.35 ± 0.10     |
| SOS           | 4                      | 2.02 ± 2.17     |
| P-GFLG-SOS    | 4                      | 32.9 ± 4.94     |
| P-(GFLG-Mce6)−Fab′ | 4                   | 43.8 ± 2.90     |

* Dm is the median-effect dose that inhibits the cell growth by 50%. IC50 and Dm values are the means ± SEM (n = 6 in single experiment).

interesting to note the enormous difference in the activity of SOS toward OVCAR-3 cells and toward the human A498 renal cell line.31 Our previous study on human renal A498 cells demonstrated that free SOS and P-GFLG-SOS conjugates were effective individually and in combination with free DOX and P-GFLG-DOX or free Mce6 and P-GFLG-Mce6, respectively.31 The IC50 values for Mce6 toward both cell types (OVCAR-3 and A49831) were similar. However, SOS was very effective toward A498 cells (IC50 = 3 nM),31 but its activity toward OVCAR-3 was about 670 times lower. These data are consistent with the results of the NCI anticancer drug screen consisting of a panel of 60 human cancer cell lines. Rivera et al. showed that after a 2-day continuous exposure to SOS, OVCAR-3 cells were less sensitive to SOS than the A498 cells.35 These results reflect the different p53 status in these cell lines. The inhibitory effect of SOS is mediated through p53; the disruption of the p53-HDM-2 interactions results in increasing p53 accumulation in tumor cells.33 It was demonstrated that the p53 status of A498 cells and OVCAR-3 cells is wild-type and mutant, respectively.59 However, the study of the p53 gene in human ovarian carcinoma cell lines by Yaginuma and Westphal showed that the wild-type p53 protein was detectable in OVCAR-3 cells by immunoprecipitation analysis.60 These reports indicated that OVCAR-3 cells can be inhibited by higher concentrations of SOS, but have a considerably lower sensitivity when compared to the A498 cell line.

The IC50 doses of nontargeted conjugates, P-GFLG-Mce6 and P-GFLG-SOS, were higher than those of free Mce6 and SOS, respectively. These results reflect the different mechanisms of cell entry of free drugs vs copolymer conjugates.61 In contrast, the targeted P-(GFLG-Mce6)−Fab′ conjugate was 2 and 9 times more effective than Mce6 and P-GFLG-Mce6, respectively. The cytotoxicity data with Mce6 conjugates were in agreement with biorecognition data and internalization mechanisms (Figures 1, 2 and 3). There was a discrepancy in data obtained for P-(GFLG-SOS)-Fab′. This conjugate showed faster internalization (Figure 1) and moderately better biorecognition (Figure 2) than P-GFLG-SOS; however, it possessed a slightly weaker inhibitory effect than P-GFLG-SOS (Table 3). One explanation may be in the long drug exposure time. After long exposure times the intracellular drug content of targeted and nontargeted conjugates may be similar.62 Furthermore, the efficacies of SOS and its conjugates might be limited by the problem of SOS trafficking to the subcellular compartments where p53 is

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The basis for the difference between mAbs used in the study of Mawby et al. and Boerman et al. or Campbell et al. is unknown and deserves further investigation. One possibility is that unusual amino acid sequence in the epitope by two monoclonal antibodies differing mainly in their heavy chain variable sequences. Clin. Exp. Immunol. 1994, 98 (1), 95–103.

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Table 4. Dose Ratios and IC50 Doses in Combinations of Free Drugs (SOS+Mce6), Nontargeted Copolymer Conjugates (P-GFLG-SOS+P-GFLG-Mce6), and Targeted Copolymer Conjugates [P-(GFLG-SOS)-Fab′+P-(GFLG-Mce6)-Fab′] in OVCAR-3 Cells

| drug combination | drug A | drug B | dose ratio | Dm (μM) | IC50 (μM) |
|------------------|--------|--------|------------|---------|-----------|
| SOS Mce6         | 1:1.61 | 0.096 + 0.0077 (0.027 + 0.0095) |
| P-GFLG-SOS P-GFLG-Mce6 | 1:0.38 | 1.69 + 0.22 (1.22 + 0.47) |
| P-(GFLG-SOS)-Fab′ P-(GFLG-Mce6)-Fab′ | 1:0.031 | 1.70 + 0.38 (1.65 + 0.051) |

In Vitro Inhibition of OVCAR-3 Cell Growth with Drug Combinations. The investigation of possible synergistic, additive, or antagonistic effects of sequential combinations of SOS+Mce6, P-GFLG-SOS+P-GFLG-Mce6, or P-(GFLG-SOS)-Fab′+P-(GFLG-Mce6)-Fab′ against the ovarian carcinoma OVCAR-3 cell line was performed in vitro by exposing cells to SOS or its conjugates for 4 days, followed by exposure of cells to Mce6 or its conjugates for 1 day, and finally, a 30 min irradiation. This sequential combination was chosen because the optimal exposure times of SOS/P-GFLG-SOS/P-(GFLG-SOS)-Fab′ and Mce6/P-GFLG-Mce6/P-(GFLG-Mce6)-Fab′ were different (4 days for SOS and 1 day for Mce6), as mentioned above. The dose ratios of each combination (Table 4) were based on their respective IC50 concentrations from Table 3 as a series of 2-fold dilutions from 4 to 0.03125 times IC50. Figure 4 shows the composite dose–response curves and median-effect plots of OVCAR-3 cells, indicating the antiproliferative effects of single agents and their combinations. The dose–response curves for combined treatment were obtained by plotting % cell viability (y) vs the combined dose of two single agents (x). The median-effect plots of single agents and combinations were derived from the linear part of dose–response curves. All of the combination treatments showed antiproliferative activities toward OVCAR-3 cells. The dose ratio and Dm values of the combination treatments are shown in Table 4. The IC50 dose of each drug in combinations was significantly lower than those of each drug as single agents (compare Tables 3 and 4). These results clearly indicate that all of the combination treatments were effective against OVCAR-3 cells.

The CI analysis was used to assess the drug–drug interaction of the sequential combinations of free drugs, nontargeted and targeted copolymer conjugates toward OVCAR-3 cells in vitro. In the CI analysis, values of CI < 1, CI = 1, and CI > 1 indicate synergy, additivity, and antagonism, respectively. Figure 5 shows the combination index plots (f5−CI plots) over all inhibition effect levels (f5 = 0.05–0.95 or 5–95% of inhibition effect) in OVCAR-3
The sequential combinations of SOS+Mce6 and P-GFLG-SOS+P-GFLG-Mce6 yield CI values lower than 1 over the entire range of cytotoxicity, indicating very strong synergistic to synergistic effects. The P-(GFLG-SOS)-Fab′+P-(GFLG-Mce6)-Fab′ combination also displayed a strong synergism for $f_a$ values up to about 0.85, but showed synergistic effect and nearly additive effect at $f_a = 0.9$ and 0.95, respectively.

The drug interactions may depend on the differences of drugs in the combination, such as physicochemical properties, the mechanisms of action, and the drug exposure schedules. All these differences may result in different antitumor activities. SOS and Mce6 are hydrophobic low molecular weight molecules, but both have different mechanisms and sites of action. SOS acts on p53 and DNA while Mce6 can cause damage to biological molecules by generation of reactive oxygen species. In our previous study, we compared the simultaneous combination of P-GFLG-SOS+P-GFLG-Mce6 to SOS+Mce6 against the human renal A498 carcinoma cell line. After 16 h cell exposure to the combinations, both combinations displayed synergism for $f_a$ up to 0.8, but showed slight antagonism and near additivity at $f_a = 0.95$. Many researchers have studied the antitumor activities following different drug exposure schedules. For example, the simultaneous and sequential exposures of irofulven with oxaliplatin or cisplatin against human breast, colon, and ovarian cancer cell lines showed that the sequence oxaliplatin...
followed by irofulven displayed better synergistic effect than the other schedules.68

Conclusion

Combination chemotherapy and PDT with free SOS and Mce6, their nontargeted and Fab′-targeted HPMA copolymer conjugates in human ovarian carcinoma OVCAR-3 cells was evaluated. Sequential combinations of these therapeutics produced very strong synergism to nearly additivity in the treatment of OVCAR-3 cells. The synergistic effects ranked in the order P-GFLG-SOS+P-GFLG-Mce6 > SOS+Mce6 > P-(GFLG-SOS)-Fab′+P-(GFLG-Mce6)-Fab′. These data support continued in vivo investigations of SOS and Mce6 combinations to determine the antitumor activity for the treatment of ovarian cancer.

Abbreviations Used

AIBN, 2,2′-azobisisobutyronitrile; APMA, N-(3-aminopropyl)methacrylamide hydrochloride; CI, combination index; D₅₀, median-effect dose; DI, deionized; DIPEA, N,N′-disopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, N,N-dimethylformamide; DOX, doxorubicin; EPR, enhanced permeability and retention; GFLG, glycyphenylalanyleucylglycine; HPMA, N-(2-hydroxypropyl)methacrylamide; IC₅₀, concentration that inhibited cell growth by 50% as compared with control cell growth; MA, methacryloyl; mAb, monoclonal antibody; Mₘ, weight average molecular weight; Mce6, mesochlorin e₆ monoethylenediamine disodium salt; MTT assay, modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; ONp, p-nitrophenoxy; P, HPMA copolymer backbone; PDT, photodynamic therapy; P-GFLG-Mce6, HPMA copolymer–Mce6 conjugate; P-(GFLG-Mce6)-Fab′, antibody Fab′ fragment targeted HPMA copolymer–Mce6 conjugate; P-GFLG-ONp, HPMA copolymer precursor containing reactive p-nitrophenyl ester groups at side chain termini; P-GFLG-SOS, HPMA copolymer–SOS conjugate; P-(GFLG-SOS)-Fab′, antibody Fab′ fragment targeted HPMA copolymer–SOS conjugate; 5-SFX, 6-(fluorescein-5-carboxamido)hexanoic acid succinimidyl ester; SMCC, succinimidyl trans-4-(maleimidomethyl)cyclohexane-1-carboxylate; SOS, 2,5-bis(5-hydroxymethyl-2-thienyl)furan; THF, tetrahydrofuran.

Acknowledgment. We thank the Drug Synthesis and Chemistry Branch of National Cancer Institute for supplying SOS. The research was supported in part by the NIH grant CA51578 from the National Cancer Institute, and by the Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0176/2545).

MP800006E

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