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ORIGINAL ARTICLE

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Enhancing the anticancer efficacy of camptothecin using biotinylated poly(ethyleneglycol) conjugates in sensitive and multidrug-resistant human ovarian carcinoma cells

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Abstract *Background*: Camptothecin (CPT) is an anticancer agent that kills cells by converting DNA topoisomerase I into a DNA-damaging agent. Although CPT and its derivatives are now being used to treat tumors in a variety of clinical protocols, the low water solubility of the drug and its unique pharmacodynamics and reactivity in vivo limit its delivery to cancer cells. To increase the anticancer efficacy of CPT a special drug delivery system is needed. *Purpose*: To synthesize a novel camptothecin-poly(ethylene glycol) conjugate (CPT-PEG) which includes biotin as a moiety to enhance nonspecific and/or targeted uptake via the sodium-dependent multivitamin transporter (SMVT) and to evaluate its anticancer activity and apoptosis induction. *Methods*: CPT-PEG and CPT-PEG-biotin conjugates were synthesized and studied in vitro in A2780 sensitive and A2780/AD multidrug-resistant human ovarian carcinoma cells. Cytotoxicity, apoptosis induction, expression of genes encoding BCL-2 and apoptotic protease-activating factor 1 (APAF-1) proteins and caspases 3 and 9 as well as caspase activity were measured. *Results*: We found that the conjugation of CPT with a simple linear PEG polymer led to a more than 12-fold enhancement of CPT toxicity in both sensitive and multidrug-resistant cells. Biotinylation of the PEG led to a further increase in CPT toxicity (5.2 times in sensitive and 2.1 times in multidrug-resistant cells) compared to the nonbiotinylated CPT-PEG conjugate. As a result, the cytotoxicity of the CPT-PEG-biotin conjugate increased more than 60 times in sensitive and almost 30 times in resistant cells, probably by enhancing nonspecific passive and/or SMVT-mediated uptake. In contrast, the same amounts of PEG and PEG-biotin conjugates without CPT did not induce cell death in either sensitive or resistant cells. Further analysis showed that the biotinylated CPT-PEG conjugate induced apoptosis more significantly than the same equivalent concentrations of free CPT or nonbiotinylated CPT-PEG. The enhancement of proapoptotic activity was achieved by the overexpression of genes encoding the APAF-1, and caspases 3 and 9, increasing caspase activity and simultaneously downregulating the BCL-2 gene. *Conclusions*: The results obtained demonstrate that the binding of CPT to PEG/PEG-biotin polymers increases its cytotoxicity, ability to induce apoptosis by the activation of caspase-dependent cell death signaling pathway and simultaneous suppression of antiapoptotic cellular defense. This suggests that the targeting approach utilizing transporters such as SMVT may substantially improve the delivery of CPT and its anticancer activity by enhancing cellular permeability and possibly retention of CPT.

Keywords Camptothecin · Poly(ethyleneglycol)-biotin conjugates · Sodium-dependent multivitamin transporter · Apoptosis · Human ovarian carcinoma

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Introduction

Camptothecin (CPT), a plant alkaloid derived from *Camptotheca acuminata* (also known as *xi shu* or Chinese Happy tree), is an anticancer agent that causes DNA damage by targeting DNA topoisomerase I [17]. CPT reversibly stabilizes the covalent enzyme-DNA intermediate in which DNA topoisomerase I is linked via a tyrosine residue in the protein to the 3′-phosphoryl end of the broken DNA strand [12]. The resulting reversible single strand nick might be converted into an irreversible lethal lesion, probably a double strand break, upon encountering the damaged DNA with a DNA replication fork [43]. If the resulting double strand break is not repaired, cell death results.

CPT has low water solubility, unique pharmacodynamics and reactivity in vivo, with respect to both drug hydrolysis and blood-protein interactions. These factors have confounded its pharmaceutical development and clinical utilization [3, 14]. A variety of different strategies are being used to modulate the systemic delivery of this class of agents in order to increase solubility, antitumor activity and/or reduce undesirable side effects. These approaches include, but are not limited to, the development of water- and lipid-soluble prodrugs and drug derivatives, polymer-bound derivatives, tumor-targeted long-circulating liposomes, aerosol liposomal formulations, and alternative routes of administration (intrathecal, intraperitoneal, local) [3, 15, 18, 23, 25, 40, 42].

It has been found that linking of a drug to high molecular weight polymers enhances solubility and improves distribution to the tumor through enhanced permeability and retention (EPR) [11, 24, 30, 29, 40]. However, enhanced uptake into tumor cells is unlikely given that much smaller PEGs are not able to penetrate cell membranes. If tumor cell uptake can be enhanced by means of a specific mechanism of internalization of water-soluble polymer-dug conjugates via endocytosis, the conjugate may bypass existing multidrug resistance mechanisms of cancer cells and prevent the de novo development of resistance [26, 27].

Other pharmaceutical shortcomings of CPT, including difficulty in formulation and instability of the active lactone form due to interactions with human albumin, might also be improved by conjugation to polymers. Different types of CPT conjugates including poly-(L-glutamic acid)-CPT [39, 41], HPMA copolymer-CPT [4, 11], poly(ethylene glycol)-CPT (CPT-PEG) [7, 8, 11], carboxymethyl dextran-CPT conjugate [33], have been recently used for these purposes. Because of the endocytotic mechanism of internalization, in most cases polymer-bound drugs have less toxicity and a slower cellular uptake rate compared to the free drugs [10, 26, 28].

We have shown that the incorporation of certain chemical features (i.e., targeting moieties) into a macromolecule can enormously enhance the rate of uptake of these conjugates into cells [37, 38]. Such moieties can enhance adherence to the plasma membrane being internalized and/or facilitate receptor-mediated endocytosis or act as substrates for known active transporters [19]. Several types of targeting moieties might be used for this purpose including antibody or antibody fragments [19, 20, 21], luteinizing hormone-releasing hormone (LHRH) targeted to the LHRH receptor expressed by ovarian cancers [1], different fusion proteins/sequences [2, 16, 31], lipid, sugars, liposaccharides and small molecular weight ligands to target specific uptake mechanisms or receptors [36, 37, 44].

More recently attention has been directed to the sodium-dependent multivitamin transporter (SMVT) that is responsible for the transfer of the vitamins pantothenate and biotin and the essential metabolite lipoate into many cell types [6, 35, 36]. We have used biotin to target the SMVT to improve the intestinal absorption of peptides [36] and have shown that PEG-biotin conjugates may interact with the SMVT transporter [37]. We have also studied the relationship between the optimal molecular descriptors of biotin conjugates and cell uptake properties in order to design effective drug delivery vehicles [37]. Based on the results of this study, we hypothesized that targeting biotinylated CPT-PEG conjugates to the SMVT would enhance the anticancer activity of the parent drug. The present work was designed to test this hypothesis and study cytotoxicity, apoptosis induction and the apoptosis-signaling pathway of biotinylated CPT-PEG conjugates in sensitive and multidrug-resistant human ovarian carcinoma cells, however, direct interaction studies with SMVT have not yet been performed.

Materials and methods

Chemicals

CPT was obtained from Sigma Chemical Company (St. Louis, Mo.), t-boc glycine was obtained from Bachem (King of Prussia, Pa.), PEG-NHS (molecular weight about 5000) and biotin-PEG-NHS (molecular weight about 3400) were obtained from Shearwater Corporation (Huntsville, Ala.), and all other chemicals were purchased from Sigma or Fisher Scientific (Fair Lawn, N.J.) and used as received.

The conjugates used in this study were synthesized using a two-step procedure (Fig. 1) modified from the method used by Greenwald et al. [13]. Briefly, t-boc-glycine (0.31 g, 1.71 mmol) was dissolved in 20 ml anhydrous methylene chloride at room temperature and to this solution were added DIPC (1,3-diisopropylcarbodiimide, 267.7 μl, 1.71 mmol), DMAP (4-dimethylaminopyridine, 0.14 g, 1.14 mmol) and CPT (0.20149 g, 0.57 mmol) at 0°C. The reaction mixture was allowed to warm to room temperature and left overnight. The product was washed with 0.1 *N* HCl, dried and evaporated under reduced pressure to yield the solid product. The product was recrystallized from methanol to give CPT-glycinate. The t-boc protection group was removed by dissolving the CPT-glycinate in a mixture of methylene chloride and TFA (50:50) and stirring at room temperature for 3 h. Solvent was evaporated under reduced pressure and the solute precipitated using ether to give CPT-glycinate·TFA salt. CPT-glycinate·TFA salt (34.0 mg or 50 mg), PEG-NHS or biotin-PEG-NHS (100 mg) were added to methylene chloride (5 ml) and *N*,*N*-diisopropylethylamine (50 μl) was added to adjust the pH to basic. The reaction was

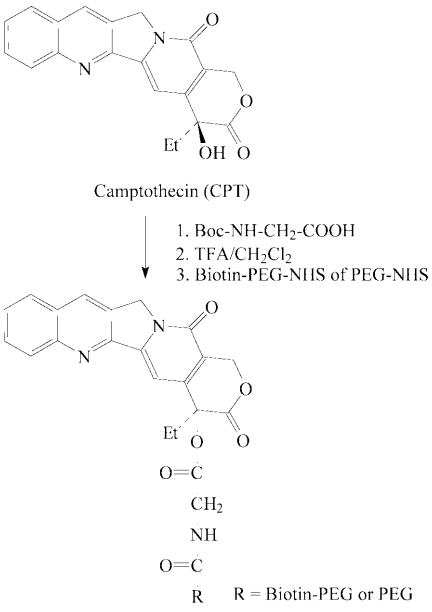

Fig. 1. Schemes of synthesis CPT-PEG conjugates

stirred for 3 h at room temperature. The product was recrystallized from cold ether and dried under vacuum overnight.

Conjugate structures were confirmed using several methods. First, mass spectrometry of the conjugates demonstrated a peak at the expected molecular weight using MALDI/TOF (Voyager System 6080; PE Biosystems, Foster City, Calif.). Second, reversed-phase HPLC analysis of the unconjugated CPT, and CPT-PEG and CPT-PEG-biotin showed different retention times (about 7 min for unconjugated CPT and about 5.5 min for the conjugates). Third, HPLC analysis using a size exclusion column demonstrated a unique peak at about 11 min for CPT-PEG and CPT-PEG-biotin, but unconjugated CPT did not elute even after 1 h. Finally, we observed dramatic increases in water solubility of these conjugates over unconjugated CPT.

Because of the low aqueous solubility of CPT water solutions, the drug was dissolved in dimethyl sulfoxide (DMSO). To prepare working solutions, the stock was diluted with medium to keep the final concentration of DMSO less than 1%. Preliminary experiments showed that such concentrations of DMSO did not have a significant effect on the cells. CPT-PEG and CPT-PEG-biotin conjugates (both extremely water soluble) were dissolved in phosphate-buffered saline (pH 7.4) and diluted with medium before use.

Cell culture

The human ovarian carcinoma cell lines, A2780 (sensitive) and A2780/AD (multidrug-resistant) were obtained from Dr. T.C. Hamilton (Fox Chase Cancer Center, Pa.). A2780/AD is a multidrug-resistant cell line [9, 26] that expresses the MDR1 gene encoding P-glycoprotein (Fig. 2). Both A2780 and A2780/AD cell lines express the SMVT gene encoding SMVT and the *MRP* gene encoding multidrug resistance-associated protein. The latter is expressed more significantly in A2780/AD multidrug-resistant cells. Cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 (v/v) in air. All experiments were performed on cells in the exponential growth phase.

Cytotoxicity assay and apoptosis detection

The cytotoxicity of drugs was assessed using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described [26]. A modified MTT assay is being routinely used in our laboratory to measure cytotoxicity of different drugs in cell culture experiments. This method allows testing of drugs in a wide range of concentrations (2^{10}–2^{44} relative units) with acceptable reproducibility and quantitative analysis using a conventional microtiter plate reader. The method of calculation and corresponding computer program have been developed and extensively tested [10, 19, 20, 21, 26, 27, 28].

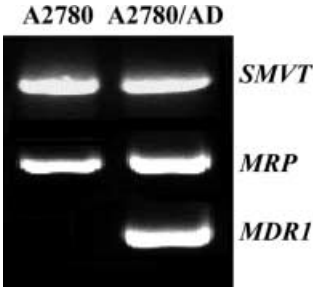

Fig. 2. Typical gel electrophoresis images of RT-PCR products of genes encoding SMVT, multidrug-associated protein (*MRP*) and P-glycoprotein (*MDR1*) in A2780 sensitive and A2780/AD multidrug-resistant cells

Apoptosis induction was analyzed in A2780 sensitive and A2780/AD multidrug-resistant human ovarian carcinoma cells separately incubated with free CPT, CPT-PEG conjugate, CPT-PEG-biotin conjugate. The concentrations of CPT (free and conjugated) were 5 n*M* for sensitive cells and 10 n*M* for resistant cells. Two approaches were used to assess apoptosis induction. The first approach was based on the measurement the enrichment of cell cytoplasm by histone-associated DNA fragments (mono- and oligonucleosomes) using anti-histone and anti-DNA antibodies by a cell death detection ELISA Plus kit (Roche, Nutley, N.J.) as previously described [28]. The method was used to analyze time-dependent apoptosis induction (0, 6, 12, 18, 24, 36, 44, 72 h) in A2780 and A2780/AD cells. The second approach was based on the detection of single- and double-stranded DNA breaks (nicks) by an in situ cell death detection kit (Roche) using the terminal deoxynucleotidyl transferase mediated dUTP-fluorescein nick end labeling (TUNEL) method as previously described [28]. After 72 h of incubation with drugs, cells were fixed and permeabilized and incubated with the TUNEL reaction mixture. The label incorporated at the damaged sites of the DNA was visualized using a fluorescence microscope.

Gene expression

Reverse transcription (RT) combined with polymerase chain reaction (PCR) was used for the analysis of gene expression in cells incubated with drugs for 72 h. The concentrations of CPT (free and conjugated) were 5 n*M* and 10 n*M* for sensitive and multidrug-resistant cells, respectively. Total cellular RNA was isolated using an RNeasy kit (Qiagen, Valencia, Calif.). First-strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (Pharmacia, Kalamazoo, Mich.) with 1 μg total cellular RNA (from 1×10^7 cells) and 100 ng random hexadeoxynucleotide primer (Pharmacia). After synthesis, the reaction mixture was immediately subjected to PCR, which was carried out using a GenAmp PCR System 2400 (Perkin Elmer, Shelton, Ct.). The pairs of primers used to amplify each type of cDNA and the PCR regimes are detailed in Table 1. PCR products were separated in 4% NuSieve 3:1 Reliant agarose gels (BMA, Rockland, Me.) in 1×TBE buffer (0.089 M Tris/borate, 0.002 *M* EDTA, pH 8.3; Research Organics, Cleveland, Ohio) by submarine electrophoresis. The gels were stained with ethidium bromide and digitally photographed using a Gel Documentation System 920 (Nucleo-Tech, San Mateo, Calif.).

Table 1. List of primers (from 5′ to 3′) and PCR regimens used for RT-PCR

No.	Gene	PCR primers	PCR regimen[a]
1	*SMVT*	CTG TCC GTG CTG GCC CTG GGC GAC CAG GCC AAT GAG GCA GCC	1
3	*BCL-2*	GGA TTG TGG CCT TCT TTG AG CCA AAC TGA GCA GAG TCT TC	2
4	*APAF-1*	GGG TTT CAG TTG GGA AAC AA CAC CCA AGA GTC CCA AAC AT	2
5	*CASP3*	TGG AAT TGA TGC GTG ATG TT GGC AGG CCT GAA TAA TGA AA	2
6	*CASP9*	TGA CTG CCA AGA AAA TGG TG CAG CTG GTC CCA TTG AAG AT	2
7	β_2-*m* (internal standard)	ACC CCC ACT GAA AAA GAT GA ATC TTC AAA CCT CCA TGA TG	2

[a] *1* 94°C/30 s, 55°C/1 min, 72°C/1 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 s, 72°C/1 min for 28 cycles, 60°C for 10 min

Caspase activity

Direct measurements of caspase activity were made using colorimetric protease assay kits (PanVera, Madison, Va.) as previously described [32] after the incubation of cells with CPT, CPT-PEG and CPT-PEG-biotin within 72 h. The concentrations of CPT (free and conjugated) were 5 n*M* and 10 n*M* for sensitive and multidrug-resistant cells, respectively. The assay is based on the spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the substrates X-*p*NA, where X stands for the amino acid sequence recognized by the specific caspase (DEVD and LEHD for caspases 3 and 9, respectively). The increase in caspase activity was determined by comparing these results with the level of the untreated control incubated with saline.

Results

Cytotoxicity of CPT, CPT-PEG and CPT-PEG-biotin

The measurement of cytotoxicity of free CPT and CPT-PEG, CPT-PEG-biotin conjugates showed that the conjugation of CPT with a simple linear PEG polymer led to a greater than 12 times enhancement in CPT toxicity in both sensitive and multidrug-resistant cells (Fig. 3). Biotinylation of CPT-PEG led to a further increase in drug toxicity (5.2 times in sensitive and 2.1 times in multidrug-resistant cells). As a result, the cytotoxicity of the CPT-PEG-biotin conjugate was increased more than 60 times in sensitive and almost 30 times in resistant cells when compared with free CPT. In contrast, the same amounts of PEG and PEG-biotin conjugates without CPT did not induce cell death in either sensitive or resistant cells (data not shown).

Apoptosis induction

Figure 4 shows apoptosis induction in A2780 sensitive and A2780/AD multidrug-resistant cells by CPT and the CPT conjugates assessed by the measurement of histone-associated DNA fragments (mono- and oligonucleosomes) generated in apoptosis. The results obtained in these experiments demonstrated that the conjugation of CPT with the simple linear PEG polymer significantly increased its proapoptotic activity. After the treatment of sensitive cells with the CPT-PEG conjugate within 44–72 h the degree of apoptosis induction was 1.8–2.1 times higher than the same equivalent concentration of free CPT. Further enhancement was achieved by using biotinylated PEG conjugate which induced apoptosis 2.0–2.6 times more than free drug under the same experimental conditions. A more pronounced difference between free CPT and both conjugated forms of the drug was observed in multidrug-resistant cells: 3.2 and

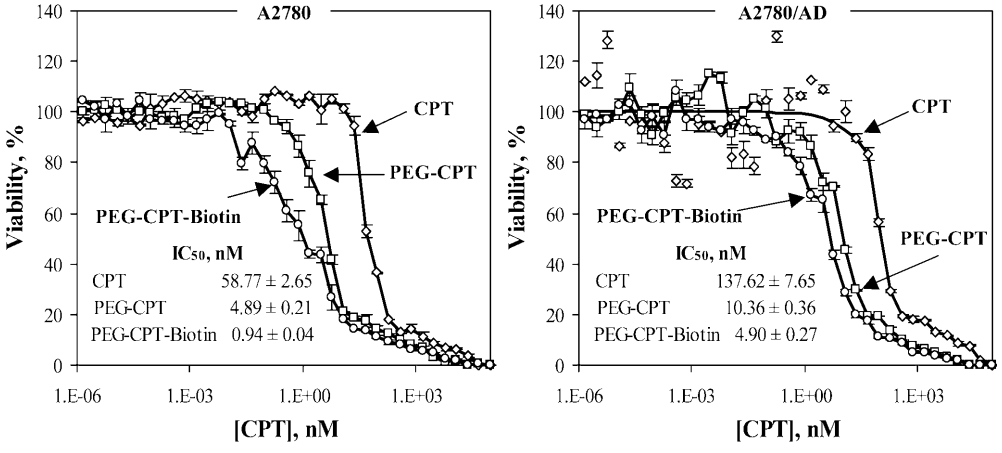

Fig. 3. Influence of CPT, and the CPT-PEG and CPT-PEG-biotin conjugates on the viability of the sensitive A2780 and multidrug-resistant A2780/AD human ovarian carcinoma cells. *Inserts* show the IC_{50} doses (drug concentrations which inhibit growth by 50% relative to nontreated control cells) of the used drugs. Means ± SD are shown

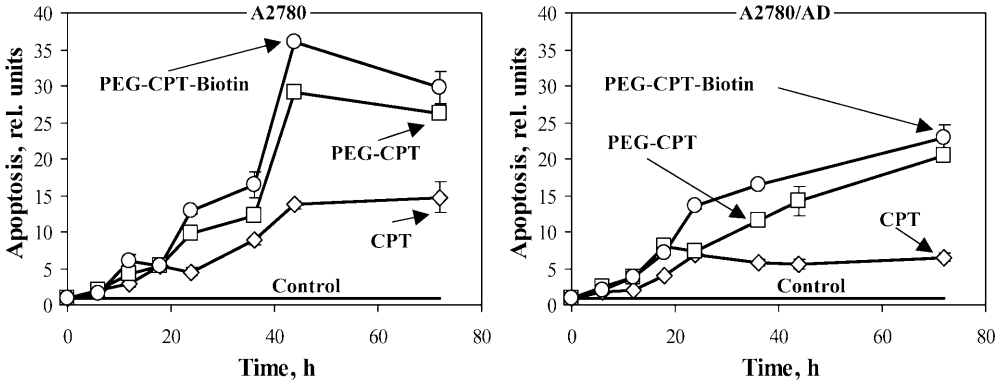

Fig. 4. Time-dependent apoptosis induction by CPT, and the CPT-PEG and CPT-PEG-biotin conjugates in sensitive A2780 and multidrug-resistant A2780/AD human ovarian carcinoma cells. The CPT concentrations were 5 n*M* (A2780) and 10 n*M* (A2780/AD). The enrichment of control cells by histone-associated DNA fragments (mono- and oligonucleosomes) was set to 1 unit and the degree of apoptosis is expressed in the relative (to control) units. Means ± SD are shown

3.5 times for CPT-PEG and CPT-PEG-biotin, respectively. These results were confirmed using another method of apoptosis detection – TUNEL labeling of single and double-stranded DNA breaks in earlier stages of DNA damage in apoptotic cells (Fig. 5). Once again, the number of apoptotic cells increased after treatment with all compounds, and this increase was significantly higher for PEG-conjugated CPT, especially for the biotinylated conjugate.

Gene expression and caspase activity

To study the influence of CPT and the CPT conjugates on apoptosis signaling pathways, A2780 sensitive and A2780/AD multidrug-resistant cells were incubated with 5 and 10 n*M* (A2780 and A2780/AD cells, respectively) of CPT, CPT-PEG and CPT-PEG-biotin conjugates for 72 h. Expression of genes encoding BCL-2, APAF-1 proteins and caspases 3 and 9 were assessed by RT-PCR. All drugs upregulated the expression of genes encoding APAF-1 and caspases 3 and 9, and downregulated the expression of the BCL-2 gene, but did not change the expression of the internal standard (Fig. 6). However, the degree of change was different for free and conjugated CPT. Conjugation to PEG significantly increased the expression of *APAF1, CASP3,* and *CASP9* genes while the biotinylation of PEG led to a further increase. Similarly, the downregulation of the *BCL-2* gene was more pronounced after the action of CPT-PEG-biotin conjugate.

Direct measurement of caspase activity (Fig. 7) confirmed the results of the gene expression analysis. It was found that all drugs increased activity of caspases 3 and 9. However, the activity of caspases was higher after incubation with the CPT-PEG conjugate. Biotinylation of CPT-PEG conjugate led to a further increase in the activity.

Discussion

Two main conclusions can be drawn from the present study. First, the conjugation of CPT with PEG/PEG-biotin polymers significantly enhanced CPT cytotoxicity, while both conjugates without the drug were not

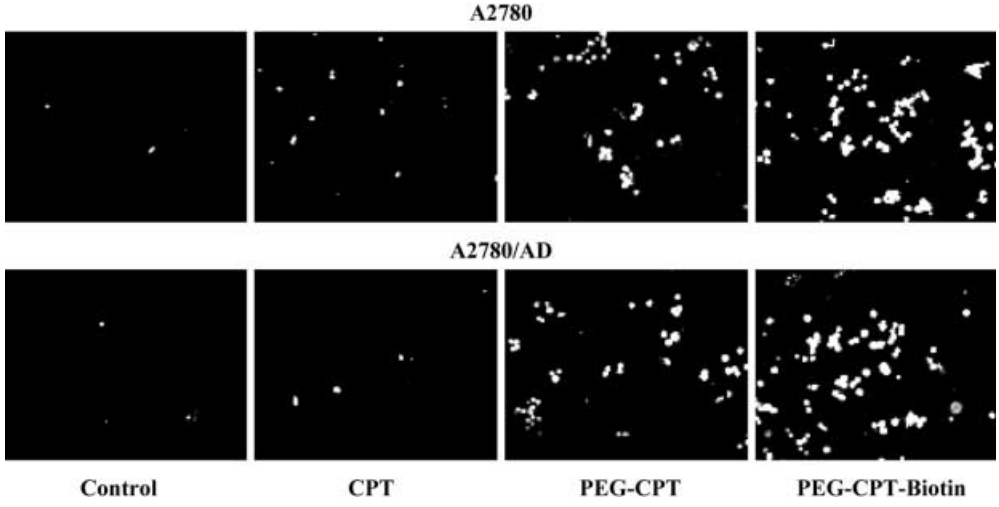

Fig. 5. Typical fluorescence microscopy images of TUNEL-labeled sensitive A2780 and multidrug-resistant A2780/AD human ovarian carcinoma cells after exposure to CPT, CPT-PEG and CPT-PEG-biotin. Cells were incubated for 72 h with CPT at concentrations of 5 n*M* (A2780) and 10 n*M* (A2780/AD)

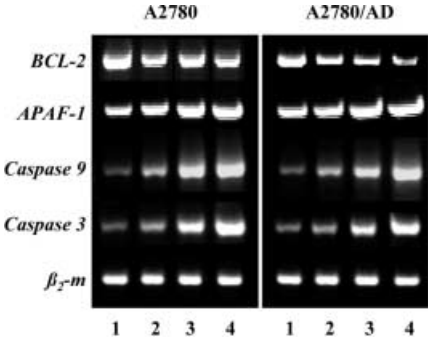

Fig. 6. Typical images of gel electrophoresis of RT-PCR products of genes encoding BCL-2 and APAF-1 proteins and caspases 9 and 3 in sensitive A2780 and multidrug-resistant A2780/AD human ovarian carcinoma cells in control (*lane 1*) and after exposure to CPT (*lane 2*), and CPT-PEG (*lane 3*) and CPT-PEG-biotin (*lane 4*) conjugates. β_2-Microglobulin (β_2-*m*) gene was used as an internal standard. Cells were incubated for 72 h with CPT at concentrations of 5 n*M* (A2780) and 10 n*M* (A2780/AD)

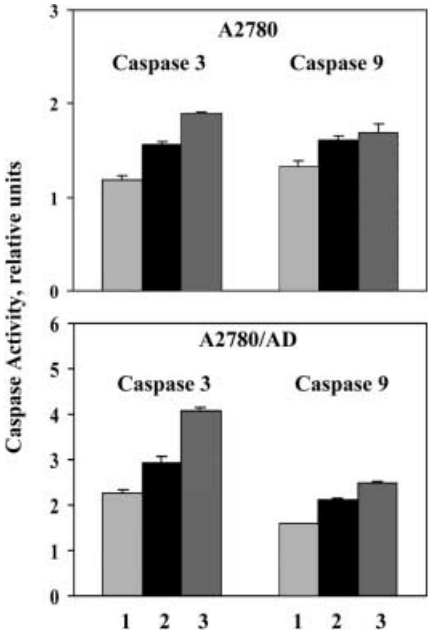

Fig. 7. Influence of CPT (*1*), CPT-PEG (*2*) and CPT-PEG-biotin (*3*) on the activity of caspases 3 and 9 in sensitive A2780 and multidrug-resistant A2780/AD human ovarian carcinoma cells. Activities in the untreated cells were set to 1 relative unit. Means ± SD are shown

toxic. These results confirm good biocompatibility of the PEG conjugates and demonstrate that the binding of CPT to PEG/PEG-biotin polymers increases its cytotoxicity probably by enhancing nonspecific passive and SMVT-mediated uptake. While we have previously confirmed both mechanisms for biotinylated PEG conjugates, to our knowledge this has not been demonstrated for nontargeted PEG conjugates. In fact, PEGs similar to those used in this study have a well-documented history of serving as nonabsorbable markers due to their inability to penetrate cell membranes [22, 41]. Therefore, it is likely that the enhancement of nonbiotinylated PEG-CPT activity was due to enhanced solubility and stability of CPT. This suggests that the targeting approach utilizing transporters such as SMVT may substantially improve the delivery of CPT.

However, several points need to be mentioned. First, the IC_{50} doses for all compounds were higher in A2780 multidrug-resistant cells than in A2780 sensitive cells. Nevertheless, resistance levels for CPT derivatives observed in the present study were markedly less than those usually observed in the same cell lines with 'classical' P-glycoprotein substrates such as topoisomerase II and tubulin inhibitors [9, 26, 27]. These findings support the general opinion that CPT itself and its derivatives are probably not P-glycoprotein and MRP protein substrates [5, 9, 15, 34, 38]. Second, the increase in IC_{50} dose for the CPT-PEG-biotin conjugate in multidrug-resistant cells was significantly higher than those for free CPT and the nonbiotinylated CPT-PEG conjugate (5.8 vs 2.1–2.3 times) and the improvement in anticancer effect caused by the biotinylation of CPT-PEG polymers in resistant cells was noticeably less than in sensitive cells (2.1 vs 5.2 times). One could conclude that this phenomenon might have been caused by less-effective biotin interactions in multidrug-resistant cells compared with sensitive ones.

Simple linear PEGs, not optimal branched PEGs, were used in this study. We have previously demonstrated that branched biotinylated PEGs have much greater cellular uptake than linear biotinylated PEG conjugates [37, 38]. Taking into account approximately equal expression of the *SMVT* gene in both cell lines, the probability of this effect seems to be low. However, to further qualify this conclusion, the direct measurement of the protein and functional studies of SMVT have to be performed. If the results of such a study, which we are currently planning to perform in our laboratories, confirms the same activity of SMVT transporter in sensitive and multidrug-resistant cells, this would indicate that A2780/AD multidrug-resistant human ovarian carcinoma cells exhibit a specific mechanism of the resistance to the biotinylated CPT-PEG conjugate suggesting that further molecular optimization of the conjugate is required.

The second main conclusion from the present study is that the PEG-based bioconjugates enhance the induction of apoptosis by CPT in ovarian cancer cells. This effect was observed both in sensitive and multidrug-resistant cells and was more pronounced for the biotinylated conjugate. Previously we have shown that the induction of apoptosis by the topoisomerase II inhibitor doxorubicin and its polymeric derivatives in human ovarian carcinoma cells requires the activation of the caspase-dependent signaling pathway [32]. The cascade of this pathway starts with cytochrome *c* release from the mitochondrion into the cytosol and the formation of the so-called 'apoptosome' which includes cytochrome *c*, apoptotic protease activating factor 1 (APAF-1), dATP and inactive caspase 9 (procaspase 9). This results in the cleavage and activation of caspase 9 (initiator of apop-

tosis), which in turn activates the apoptosis executor – caspase 3. The overexpression of BCL-2 protein prevents the release of cytochrome *c* from the mitochondrion and therefore limits apoptosis induction.

The present results demonstrate that similar mechanisms of apoptosis induction might be induced by free CPT and especially by CPT-PEG conjugates. Significant upregulation of the expression of *APAF1*, *CASP3* and *CASP9* genes as well as the increase in the activity of the studied caspases support this suggestion. In addition to activation of the caspase-dependent apoptosis signaling pathway, CPT-PEG and especially CPT-PEG-biotin conjugates significantly downregulated the expression of the *BCL-2* gene. This indicates that both conjugates suppressed the activity of cellular antiapoptotic defense mechanisms.

Taken together the results show much higher cytotoxicity and apoptosis-inducing activity of CPT-PEG conjugates when compared to free CPT. Moreover, the effects of the CPT-PEG-biotin conjugate were more pronounced than those of the non-biotinylated CPT-PEG conjugate. This makes the CPT-PEG-biotin conjugate a promising novel anticancer agent.

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