Molecular Targeting of BCL2 and BCLXL Proteins by Synthetic BCL2 Homology 3 Domain Peptide Enhances the Efficacy of Chemotherapy

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ABSTRACT
Chemotherapeutic agents are known to induce programmed cell death or apoptosis. The activation of cellular antiapoptotic defense that prevents the translation of drug-induced damage into cell death is the key factor in cellular antiapoptotic resistance that decreases the chemotherapeutic effectiveness of a broad spectrum of anticancer drugs. A novel proapoptotic anticancer drug delivery system (DDS) was designed to simultaneously induce apoptosis and suppress antiapoptotic cellular defense. The system includes three main components: 1) anticancer drug camptothecin (CPT) as an apoptosis inducer, 2) synthetic BCL2 homology 3 domain (BH3) peptide as a suppressor of cellular antiapoptotic defense, and 3) poly(ethylene glycol) (PEG) polymer as a carrier. The above DDS was studied in vitro using A2780 human ovarian carcinoma cells and in vivo on nude mice bearing xenografts of human ovarian tumor. The results obtained in both series of experiments corroborate each other. They show that the designed DDS provided intracellular delivery of active components and suppressed cellular antiapoptotic defense, leading to the more pronounced induction of caspase-dependent signaling pathway of apoptosis compared with CPT alone and simple CPT-PEG conjugate. Including BH3 peptide in complex DDS decreased apoptotic cellular defense, substantially increased toxicity of the whole complex, and provided high antitumor activity. Therefore, the proposed novel multicomponent proapoptotic anticancer drug delivery system has high potential to enhance the efficacy of chemotherapy.

According to the National Center for Health Statistics, cancer is the second leading cause of death in the United States (NCHS, 2004). Despite the advances in cancer treatment and improvements in life style and health care, death rates from cancer have not changed significantly during the last 50 years. In contrast, mortality from heart disease, the leading cause of death, declined almost 2.5-fold for the same period (NCHS, 2004). Therefore, the increase in the efficacy of cancer treatment is an essential task for modern medicine. Although localized tumors can be successfully removed by surgery, the treatment of spreading or metastatic tumors requires high-dose chemotherapy. However, the efficacy of chemotherapy is limited by the rapid development of tumor resistance (Fennelly, 1995; Minko et al., 1999; Searcey and Patterson, 2004). Chemotherapeutic agents are known to induce programmed cell death or apoptosis. The activation of cellular antiapoptotic defense that prevents the translation of drug-induced damage into cell death is considered to be the key factor in cellular resistance to a broad spectrum of anticancer drugs (Minko et al., 1999; Dharap et al., 2003; Pakunlu et al., 2003, 2004). Consequently, we hypothesized that a suppression of cellular antiapoptotic defense will enhance the efficacy of chemotherapy and prevent the development of the resistance in cancer cells during treatment.

It is known that BCL2 family proteins are key players in the cellular mechanisms of apoptosis induction and defense (Gross et al., 1999; Reed, 1999). The BCL2 protein family consists of two groups of proteins with countermodulating functions: 1) the group that can suppress apoptosis if overexpressed, and 2) the group that can induce apoptosis (Reed, 1999; Lowe and Lin, 2000). Although the precise role of these proteins in apoptosis induction and development of resistance during cancer therapy remains unclear, it was found

ABBREVIATIONS: BH3, BCL2 homology 3 domain; CPT, camptothecin; PEG, polyethylene glycol; DMSO, dimethyl sulfoxide; DDS, drug delivery system; FITC, fluorescein isothiocyanate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PCR, polymerase chain reaction; APAF1, apoptotic protease activating factor 1.
that survival or death of cancer cells following an apoptotic stimulus depends on the expression ratio of antiapoptotic to proapoptotic members of the BCL2 protein family (Oltvai et al., 1993). However, data obtained on actual cancer tissues are controversial (Herod et al., 1996; Baekelandt et al., 1999; Kassim et al., 1999; Mano et al., 1999; Schuyer et al., 2001). This controversy might be explained by the fact that almost all clinical studies have been focused on separate analyses of the expression of pro- or antiapoptotic members of the BCL2 protein family, although it is the ratio that must be considered (Oltvai et al., 1993; Reed, 1999; Schuyer et al., 2001).

The BCL2 protein family is characterized by specific regions of homology termed BCL2 homology (BH1, BH2, BH3, BH4) domains. These domains are critical to the functions of these proteins, including their impact on cell survival and their ability to interact with other family members and regulatory proteins (Johnson, 1999). It was found that the BCL2 homology 3 (BH3) domain of proapoptotic proteins from the BCL2 family is responsible for the induction of apoptosis (Cosulich et al., 1997; Johnson, 1999). Furthermore, expression of small-truncated derivatives of BAK protein containing the BH3 domain was sufficient for its cell killing activity (Cosulich et al., 1997). Moreover, it was found that short synthetic peptides, corresponding to the minimal sequence of BH3 domain when bound to the antiapoptotic BCL2 family proteins, suppress the cellular antiapoptotic defense (Holinger et al., 1999; Lutz, 2000; Dharap and Minko, 2003; Dharap et al., 2003). Therefore, BH3 peptide can provide molecular targeting of antiapoptotic members of BCL2 protein family and potentially improve traditional therapy of ovarian cancer by decreasing the antiapoptotic cellular defense against anticancer drugs.

Recently, we developed a novel anticancer proapoptotic drug delivery system that contains 1) camptothecin (CPT) as the anticancer drug (apoptosis-inducing agent), 2) synthetic BH3 peptide as a suppressor of antiapoptotic cellular defense, and 3) poly(ethylene glycol) (PEG) as a carrier (Dharap et al., 2003). The in vitro examination of this system showed its high potential to suppress cellular antiapoptotic defense and increase the anticancer efficacy of CPT. Although for the purpose of the present investigations any type of water-soluble polymer could be used, PEG polymer was selected as a carrier for this system based on its high water solubility, known chemistry of conjugation with different molecules, very low toxicity and immunogenicity, and wide use and our team’s extensive experience using it. We show that PEG polymer with a molecular mass of approximately 5000 Da is the most suitable carrier for the delivery of anticancer drugs and other active components, including peptides, because it preferentially accumulates in solid tumors by an enhanced permeability and retention effect (Maeda, 2001), providing preferentially accumulates in solid tumors in vivo. The present investigations are aimed at further in vitro and in vivo evaluation of the antitumor effect of this system.

Materials and Methods

Drug, Peptide, and Synthesis of Conjugates. CPT was obtained from Sigma Chemical Co. (St. Louis, MO). The drug, insoluble in water, was first dissolved in 10% dimethyl sulfoxide (DMSO) for both in vitro and in vivo experiments. PEG (mol. wt. ~5000) was obtained from Nektar (Huntsville, AL). BH3 peptide was synthesized according to our design by American Peptide Company, Inc. (Sunnyvale, CA). The sequence of native BH3 peptide was slightly modified by adding an extra residue of cysteine at the C terminus. The amino group of glycine was linked by an amide bond to the cross-linking reagent NHS-PEG-VS (NHS, N-hydroxysuccinimide; and VS, vinylsulfone). When added to the reaction mixture, the thiol group of BH3 formed a thioether bond when reacted with the vinylsulfone group on the PEG. The modified sequence of the peptide is presented in Fig. 1. All other chemicals were purchased from Sigma Chemical Co. or Fisher Scientific (Pittsburgh, PA) and used as received. The conjugates used in this study were synthesized using a two-step procedure modified from Conover et al. (1997) and Greenwald (2001) as previously described (Dharap et al., 2003) for the two conjugates CPT-PEG and CPT-PEG-luteinizing hormone-releasing hormone. The BH3 peptide was N-acetylated and C-amidated to mimic the natural structure of the peptide backbone in the context of the protein, as well as to provide stability toward aminopeptidases and carboxypeptidases. The structures of CPT and CPT-PEG-BH3 conjugate are shown in Fig. 1.

Cell Line. The human ovarian carcinoma A2780 cell line was obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were cultured in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Cells were grown at 37°C in a humidified atmosphere of 5% CO2 (v/v) in air. All experiments were performed on cells in the exponential growth phase.

Cytotoxicity. The cytotoxicity of peptides was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (Minko et al., 1999; Pakunlu et al., 2004). To measure cytotoxicity, cells were separately incubated in microtiter plates with different concentrations of CPT, CPT-PEG, and CPT-PEG-BH3 conjugates in the cell growth medium. Medium cells received an equivalent volume of fresh medium. The duration of incubation was 24 h. Based on these measurements, IC50 doses of conjugates (the concentration of DDS necessary to inhibit the cell growth by 50%) were calculated as previously described (Minko et al., 1999; Pakunlu et al., 2004).

Intracellular Localization of BH3 Peptide. To analyze intracellular localization of BH3 peptide, the peptide was labeled by fluorescein isothiocyanate (FITC). Cancer cells were incubated 24 h with labeled BH3 peptide alone or with PEG-BH3 conjugate, which was synthesized as previously described (Dharap et al., 2003). FITC

![Fig. 1. Sequence of BH3 peptide and structures of CPT and CPT-PEG-BH3 conjugate. The conjugation site in CPT (hydroxyl at C20 position) is highlighted.](image-url)
fluorescence was visualized by fluorescence microscopy (Zeiss Axiostar Plus fluorescence microscope; Carl Zeiss Inc., Thornwood, NY) using the following filters: excitation 470/40 nm and emission 525/50 nm (FITC).

**Animal Tumor Model.** Animal model of human ovarian carcinoma xenografts was used as previously described (Minko et al., 2000; Dharap et al., 2005). Briefly, A2780 human ovarian cancer cells (2 x 10^6) were subcutaneously transplanted into the flanks of female athymic nu/nu mice. When the tumors reached a size of approximately 1 cm^3 (15–20 days after transplantation), mice were treated intraperitoneally with CPT, CPT-PEG, PEG-CPT-BH3 conjugates, or saline. The dose of all substances (10 mg/kg for the single injection) corresponded to the maximum tolerated dose of CPT-PEG. Maximum tolerated doses were estimated in separate experiments based on animal weight changes after the injection of increasing doses of drugs as previously described (Minko et al., 2000; Dharap et al., 2005). Animal weight was measured every day within 1 week after the treatment.

**Antitumor Activity.** Changes in tumor size were used as an overall marker for antitumor activity as previously described (Minko et al., 2000; Dharap et al., 2005). Tumor size was determined 6, 12, 18, 24, 36, 48, 72, and 96 h after the treatment of mice.

**Apoptosis.** Two approaches were used to assess apoptosis induction. The first approach was based on the measurement of the enrichment of histone-associated DNA fragments (mono- and oligonucleosomes) in homogenates of the tumor as previously described (Minko et al., 2000, 2002b). The second approach was based on the detection of single- and double-stranded DNA breaks (nicks) by an in situ cell death detection kit (Hoffman-La Roche, Nutley, NJ) using a terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) method as previously described (Pakunlu et al., 2004; Dharap et al., 2005). Apoptosis induction in tumor was measured 24 h after the treatment.

**Gene Expression.** Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used for the analysis in tumor tissue homogenates of expression of genes encoding BCL2 protein, caspase 3 (CASP3), and caspase 9 (CASP9) as previously described (Dharap and Minko, 2003; Pakunlu et al., 2003, 2004). RNA was isolated 24 h after the treatment using an RNeasy kit (QIAGEN, Valencia, CA). The following pairs of primers were used: BCL2: GGA TGG TGG CCT TCT TTG AG (sense), CCA AAC TGA GCA GAG TCT TC (antisense); BCLXL: ATG AAC TCT GCC GGG ATG G (sense), TGG ATC CAA GGC TCT AGG TG (antisense); CASP3: TGG AAT TGA TGC GTG ATG TT (sense), GGC AGG CCT GAA TAA TGA AA (antisense); CASP9: TGA CTG CCA AGA AAA TGG TG (sense), CAG CTG GTC CCA TTG AGG AT (antisense); and β_2-m: ACC CCC ACT GAA GAT GA (sense), ATC TTC AAA CCT CCA TGA TG (antisense). PCR products were separated in 4% NuSieve 3:1 Reliant agarose gels (BMA, Rockland, ME) in 1X TBE buffer (0.089 M Tris/borate and 0.002 M ethylenediaminetetraacetic acid, pH 8.3; Research Organics Inc., Cleveland, OH) by submarine electrophoresis. The gels were stained with ethidium bromide, digitally photographed, and scanned using Gel Documentation System 920 (NucleoTech, San Mateo, CA). Gene expression was calculated as the ratio of mean band density of analyzed reverse transcriptase-PCR product to that of the internal standard (β_2-m).

**Statistical Analysis.** Data obtained were analyzed using descriptive statistics, single factor analysis of variance, and presented as mean value ± standard deviation (S.D.) from four to eight independent measurements in separate experiments.

**Results**

**Conjugation of BH3 Peptide to PEG Polymer Provides for the Delivery of the Peptide into Cancer Cells.** To study cellular penetration of BH3 peptide, A2780 human ovarian carcinoma cells were incubated with BH3 peptide alone and BH3 peptide conjugated with PEG. BH3 peptide was labeled with FITC. Fluorescence was visualized by a fluorescence microscope. We found (Fig. 2) that incubation of cells with BH3 peptide alone resulted in low fluorescence intensity inside cancer cells. This shows that the penetration of BH3 peptide alone in cancer cells is relatively low. Therefore, a delivery system is required to effectively transfer this peptide inside cancer cells. We used a PEG-based system to enhance intracellular penetration of BH3 peptide and simultaneous delivery of this peptide with an anticancer drug—camptothecin. The fluorescence microscopy images of cells incubated with FITC-labeled BH3-PEG conjugate show substantially higher fluorescence inside cancer cells compared with nonconjugated BH3-FITC. The high level of this fluorescence testifies that PEG-conjugated BH3 enhances penetration of BH3 peptide inside cancer cells. Therefore, PEG polymer can be used as a delivery vehicle for this peptide similar to the previously used antennapedia peptide (Holinger et al., 1999; Schimmer et al., 2001; Walsh et al., 2002; Dharap et al., 2003).

**BH3 Peptide Significantly Enhances Anticancer Activity of CPT-PEG Conjugate in Vitro.** Toxicity of the proposed novel anticancer proapoptotic drug delivery system CPT-PEG-BH3 was studied in vitro using A2780 human ovarian carcinoma cells. The cells were incubated 24 h with CPT-PEG-BH3 conjugate. Free BH3, PEG, PEG-BH3 conjugate, free CPT, and CPT-PEG conjugate were used as controls. Free BH3 and PEG polymer were not toxic in concentrations used in this study. As seen from Fig. 3, bar 1, PEG-BH3 conjugate is relatively low in toxicity (high IC<sub>50</sub> dose). Data show that conjugation of CPT with PEG polymer significantly (P < 0.05) increased its cytotoxicity compared with free drug (compare bars 3 and 2 in Fig. 3). However, complex CPT-PEG-BH3 conjugate demonstrated substantially (several orders of magnitude) higher cytotoxicity compared with both free CPT and CPT-PEG conjugate. Therefore, BH3 peptide considerably improved anticancer effectiveness of CPT alone and PEGylated camptothecin.

**BH3 Peptide Substantially Enhances Antitumor Activity of CPT and CPT-PEG Conjugate in Vivo.** To evaluate antitumor activity of the proposed DDS, we treated nude mice bearing xenografts of human ovarian carcinoma...
with CPT, CPT-PEG, and CPT-PEG-BH3. Control mice received an equivalent dose of saline, free BH3, PEG, or PEG-BH3 conjugate. Tumor size was measured at 6, 12, 18, 24, 36, 48, 72, and 96 h after the injection, and its decrease after the treatment was used as an indicator of antitumor activity. Free BH3 and PEG did not influence tumor growth (Fig. 4, curves 2 and 3). Although the PEG-BH3 possessed a slight antitumor activity, no statistically significant differences in tumor size were observed after the treatment of mice with PEG-BH3 (Fig. 4, compare curves 4 and 1). It was found that conjugation of CPT to PEG led to the increase in its antitumor activity (Fig. 4). Treatment of mice with CPT-PEG-BH3 conjugate decreased tumor size much more significantly (P < 0.05) compared with CPT and CPT-PEG (Fig. 4, curve 7). Therefore, BH3 peptide substantially enhances antitumor activity of CPT and CPT-PEG conjugate.

BH3 Peptide Inhibits Antiapoptotic Defense and Enhances the Ability of CPT and CPT-PEG Conjugate to Activate Caspase-Dependent Signaling Pathways of Apoptosis in Tumor Cells. To evaluate the mechanisms of the enhancement of anticancer activity of CPT by BH3 peptide, we used tumor tissue homogenates to study the expression of genes encoding proteins BCL2 and BCLXL (the main player in cellular antiapoptotic defense), caspase 9 (the major initiator of proapoptotic signal), and caspase 3 (the main apoptosis executor). Free BH3 and PEG did not influence the expression of all the studied genes. A statistically significant decrease in the expression of BCL2 and BCLXL genes was observed after the incubation of cells with PEG-BH3 conjugate. This led to the small but statistically significant increase in the expression of caspases (Fig. 5, lane 4). The data obtained show that free CPT activated both proapoptotic caspase-dependent cellular signal and antiapoptotic defense by increasing the expression of BCL2, BCLXL, CASP9, and CASP3 genes encoding BCL2 and BCLXL proteins and caspase 9 and 3, respectively (Fig. 5, lane 5). Conjugation of CPT to PEG polymer led to more pronounced activation of all these genes (Fig. 5, lane 6). In contrast, complex CPT-PEG-BH3 substantially inhibited the expression of BCL2 and BCLXL mRNA. This led to further enhancement in the caspase-dependent cellular proapoptotic signal and overexpression of genes encoding caspase 3 and 9 and, therefore, increased apoptotic drive.

BH3 Peptide Significantly Increases Apoptosis Induction by CPT-PEG Conjugate in Tumor Cells. Direct measurement of apoptosis induction in the tumor tissue homogenates corroborates gene expression seen in Fig. 5. Apoptosis was analyzed by the measurement of the enrichment of histone-associated DNA fragments (mono- and oligonucleosomes) in homogenates of the tumor. Although statistically significant induction of apoptosis in tumor was observed after the treatment of mice with PEG-BH3 conjugate (on 27%) and free CPT (on 56%) (Fig. 6, bars 4 and 5) and more pronouncedly with CPT-PEG conjugate (Fig. 6, bar 6), an
approximately 30-fold increase in apoptosis induction was recorded after the treatment with complex proapoptotic delivery system containing CPT-PEG-BH3 (Fig. 6, bar 7). Therefore, the inclusion of BH3 peptide in the drug delivery system significantly increases its proapoptotic activity. This observation is also supported by the data obtained using another independent method of apoptosis analysis. Fluorescent microscopy analysis of the TUNEL-labeled tissue samples also showed that the induction of apoptosis in tumor tissue was most distinctly observed after the treatment of mice with CPT-PEG-BH3 (Fig. 7).

Discussion

We have designed a novel three-component proapoptotic anticancer drug delivery system that includes an anticancer drug (camptothecin) as an apoptosis inducer, polyethylene glycol polymer as a carrier, and BH3 peptide as a suppressor of cellular antiapoptotic defense. This delivery system was investigated in vitro on human ovarian cancer cells and in vivo on nude mice bearing xenografts of human ovarian carcinoma. The results obtained in vitro and in vivo corroborate each other. We found that PEG as a carrier provides for the transfer of BH3 peptide into cancer cells. Then the peptide binds to BCL2 and BCLXL proteins and limits cellular antiapoptotic defense. At the same time, the anticancer drug included in the complex proapoptotic DDS induces apoptosis through the caspase-dependent pathway. Apoptosis induction on the background of suppressed cellular defense substantially enhances cytotoxicity of the whole complex and its antitumor activity. As a result, the designed novel multicomponent system is more effective in suppressing the growth of cancer cells in both in vitro and in vivo experimental settings.

The results of the present investigation support our hypothesis that suppression of antiapoptotic cellular defense substantially increases efficiency of chemotherapy. Our present and previous data (Minko et al., 2000; Pakunlu et al., 2004) as well as literature (Gross et al., 1999; Kassim et al., 1999; Mano et al., 1999; Reed, 1999; Lutz, 2000; Schuyer et al., 2001; Tsuruo et al., 2003) show that acute and chronic treatment of cancer cells or tumors by anticancer drugs usually results in two opposite effects (Fig. 8). Most anticancer drugs induce cell death by the activation of intracellular apoptotic signals. The main event in this process is the leakage of the cytochrome c from the mitochondria to the cytoplasm, leading to the activation of caspase-executors of apoptosis. Although the whole complex process of apoptosis induction involves many steps, the activation of proapoptotic members of BCL2 protein family plays a central role in the initiation of apoptosis (Cosulich et al., 1997; Gross et al., 1999; Holinger et al., 1999; Reed, 1999; Lowe and Lin, 2000; Lutz, 2000). However, the launching of apoptosis activates cellular antiapoptotic defense, a complex process initiated by the activation of antiapoptotic members of the same protein family (Gross et al., 1999; Reed, 1999; Lutz, 2000). The overexpression of antiapoptotic proteins limits the leakage of the cytochrome c from mitochondria. Therefore, the process of programmed cell death and the final destiny of a cell, to live or to die, is controlled by the balance between the activity of the proapoptotic and antiapoptotic members of the same protein family. Based on this, we proposed to include in the anticancer drug delivery system a component that will suppress cellular antiapoptotic defense by limiting the activity of antiapoptotic members of BCL2 protein family. BH3 peptide is the suppressor incorporated in our polymeric multicomponent anticancer drug delivery system. Data obtained support our hypothesis that the inclusion of BH3 peptide in the drug delivery system enhances antitumor effect of anticancer drug.

Two factors mainly contributed to the exceptionally high antitumor effectiveness of CPT-PEG-BH3 conjugate: 1) improved BH3 peptide delivery into cancer cells, the high level of peptide permeation, and consequently, suppression of antiapoptotic cellular defense, and 2) enhanced antitumor activity of CPT. Although the exact mechanisms of the improvement of peptide penetration after conjugation of BH3 peptide with PEG polymer are unknown, we can speculate that such mechanisms are similar to previously discussed mechanisms of enhancement of activity of other active components of complex polymer-based delivery systems (Minko, et al., 2000).

Fig. 7. Typical fluorescent microscopy images of tumor tissue slides labeled by TUNEL 24 h after treatment of mice bearing xenografts of A2780 human ovarian carcinoma with saline, BH3, PEG, PEG-BH3, CPT, CPT-PEG, and CPT-PEG-BH3.
et al., 1998, 2004). Briefly, conjugation of BH3 peptide to a polymer prevents its degradation in the blood or aqueous solutions during its transfer toward tumor cells and therefore increases the concentration of the peptide in the tumor near plasma membrane of cancer cells. Such conjugation also provides a passive tumor targeting by the enhanced permeability and retention effect (Maeda, 2001). In addition, internalization and cytoplasmic transfer in membrane-limited vesicles after endocytosis of the conjugate prevents further degradation of BH3 peptide inside the cell. It seems that these mechanisms mainly attributed to the higher efficiency of conjugated BH3 compared with free peptide in the suppression of antiapoptotic activity in tumor cells. The experimental support of this conception and evaluation of mechanisms responsible for the phenomena are the tasks for our future investigations.

CPT, insoluble in water, required 10% of DMSO in cell culture media or phosphate-buffered saline for its dissolution. This creates several problems for the practical use of this drug in laboratory and clinics. First, DMSO is not allowed for clinical studies, and therefore several water-soluble forms of this drug have been developed (Slichenmyer and Von Hoff, 1990; Rahier et al., 2005; Srivastava et al., 2005; Zhang et al., 2005). Second, CPT is unstable in aqueous media, its anticancer activity drops, and it precipitates in vitro and especially in vivo. To overcome these obstacles, we as well as others used CPT polymer to increase the solubility of CPT and prevent its degradation in aqueous solutions (Minko et al., 2002a; Greenwald et al., 2003). Recent reports show that conjugation of CPT to PEG polymer enhanced stability of ester and amide bonds for lactone forms of CPT (Greenwald et al., 2003), which consequently led to the high anticancer activity of the drug (Minko et al., 2002a). Current experiments also showed higher water solubility of CPT-PEG conjugate and its enhanced antitumor action. This increase in the activity of CPT, achieved by the prevention of its precipitation and degradation after the conjugation with PEG, contributed to the high in vitro and in vivo efficiency of CPT-PEG-BH3 conjugate. However, such contribution was substantially lower compared with the suppression of cellular antiapoptotic defense. For instance, conjugation of CPT to PEG increased its in vitro toxicity by nearly 17 times and apoptosis induction in tumor tissues by 2 times. At the same time, the suppression of cellular antiapoptotic defense by BH3 peptide in complex CPT-PEG-BH3 conjugate further increased cytotoxicity of CPT-PEG by more than 40,000 times and apoptosis induction in vivo—more than 10 times. Apoptosis induction by BH3 peptide in the absence of anticancer drug involves the same mechanism as the suppression of cellular antiapoptotic defense through the BCL2-BCLXL pathway. In normal conditions in the absence of cell death signal, antiapoptotic members of BCL2 protein family (BCL2 and BCLXL) limit the cytochrome c release from the mitochondrion and are bound to the apoptotic protease activating factor 1 (APAF1), preventing the activation of procaspase 9 (Adams and Cory, 1998). BH3 protein, containing cell death domain of proapoptotic proteins BIK and BAX, binds to antiapoptotic members of BCL2 protein family and inactivates them preventing the neutralization of APAF1 and promoting the cytochrome c release from the mitochondrion. In the presence of cytochrome c released from mitochondria and ATP, APAF1 then binds to procaspase-9 and promotes its dimerization and activation by autocatalysis. Caspase-9 subsequently activates effector caspases. A similar mechanism is involved in the suppression of antiapoptotic cellular defense under the combined action of a cell death inducer—CPT and a suppressor of antiapoptotic cellular defense—BH3 peptide. Although PEG-BH3 conjugate by itself was able to induce caspase-dependent apoptosis, the degree of such induction (~1.3-fold) was substantially lower compared with anticancer drug-containing CPT-PEG-BH3 conjugate (~30-fold).

Therefore, we concluded that the main role in high antitumor activity of CPT-PEG-BH3 conjugate plays the simultaneous cell death induction by the anticancer drug CPT and limitation of cellular antiapoptotic defense by BH3 peptide through the suppression of BCL2 and BCLXL proteins.

The exceptionally high toxicity of the proposed multicomponent drug delivery system imposes potential adverse side effects on healthy organs. The main intracellular targets of this system include apoptosis induction and antiapoptotic defense. Although, BCL2 and BCLXL proteins are overexpressed in tumor cells compared with normal noncancer cells, similar mechanisms of pro- and antiapoptotic balance can be found in normal cells. Therefore, such highly toxic drug delivery system potentially might induce apoptosis in normal nontumor cells. Adverse side effects can be avoided by targeting of proposed proapoptotic drug delivery system specifically to cancer cells. This targeting can be achieved by adding to the DDS a fourth component—a targeting moiety to direct the DDS to tumor. Recently we proposed to use luteinizing hormone-releasing hormone peptide for specific targeting of drug delivery system to tumor cells (Dharap and Minko, 2003; Dharap et al., 2003, 2005). We expect that such a four-component targeted proapoptotic anticancer drug delivery system will 1) substantially enhance the efficiency of chemotherapy, and 2) prevent possible adverse side effects to healthy organs by targeting its high proapoptotic action specifically to cancer cells.

References