JPET Fast Forward. Published on November 15, 2005 as DOI: 10.1124/jpet.105.094243

JPET #94243

Molecular targeting of BCL2 and BCLXL proteins by synthetic BH3 peptide enhances the efficacy of chemotherapy

Sonia S. Dharap, Pooja Chandna, Yang Wang, Jayant J. Khandare, Bo Qiu, Stanley Stein, and Tamara Minko

Department of Pharmaceutics, Rutgers, The State University of New Jersey, Piscataway, NJ (S.S.D., P.C., Y.W., J.J.K., B.Q., S.S., T.M.)

JPET Fast Forward. Published on November 15, 2005 as DOI: 10.1124/jpet.105.094243
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Molecular targeting of BCL2 and BCLXL proteins

Corresponding author:

Tamara Minko, Ph.D.
Department of Pharmaceutics
Ernest Mario School of Pharmacy
Rutgers, The State University of New Jersey
160 Frelinghuysen Road
Piscataway, NJ 08854–8020
Phone: 732-445-3831 x 214
Fax: 732-445-3134
Email: minko@rci.rutgers.edu

The number of text pages: 28

Number of Tables: 0

Number of figures: 8

Number of references: 48

Number of words:

Abstract: 220

Introduction: 821

Discussion: 1500

The list of non-standard abbreviations:

DDS – drug delivery system; BH3 peptide – BCL2 homology 3 domain peptide.

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 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 when 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 a high potential to enhance the efficacy of chemotherapy.

Introduction

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 folds 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 prevent 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 (Dharap et al., 2003; Minko et al., 1999; Pakunlu et al., 2003; Pakunlu et al., 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 counter modulating functions: (1) the group that can suppress apoptosis if overexpressed, (2) the group that can induce

apoptosis (Lowe and Lin, 2000; Reed, 1999). Although the precise role of these proteins in apoptosis induction and development of resistance during cancer therapy remains unclear, it was found 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 (Baekelandt et al., 1999; Herod et al., 1996; 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 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 (Dharap and Minko, 2003; Dharap et al., 2003; Holinger et al., 1999; Lutz, 2000). 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 which 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 immunogenecity, wide use and extensive experience of our team. We show that PEG polymer with a molecular weight about 5,000 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 Enhanced Permeability and Retention (EPR) effect (Maeda, 2001) providing passive tumor targeting of the whole system and substantially increasing solubility and antitumor efficacy of poor water-soluble drugs such as CPT. The present investigations are aimed at further *in vitro* and *in vivo* evaluation of the antitumor effect of this system.

Material and Methods

Drug, Peptide and Synthesis of Conjugates. Camptothecin (CPT) was obtained from Sigma Chemical Co. The drug being insoluble in water, was first dissolved in 10% dimethyl sulfoxide (DMSO) for both *in vitro* and *in vivo* experiments. PEG (MW ~5000) was obtained from Shearwater Corporation. BH3 peptide was synthesized according to our design by American Peptide Company, Inc. 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 is N-hydroxysuccinimide; VS is 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 and used as received. The conjugates used in this study were synthesized using a two-step procedure modified from Refs. (Conover et al., 1997; Greenwald, 2001) as previously described (Dharap et al., 2003) for the two conjugates: CPT-PEG and CPT-PEG-LHRH. 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). Cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (HyClone). 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. The cytotoxicity of peptides was assessed using a modified MTT (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 plate with different concentrations of CPT, CPT-PEG and CPT-PEG-BH3 conjugates in the cell growth medium. Control cells received an equivalent volume of fresh medium. The duration of incubation was 24 h. Based on these measurements, IC_{50} 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 fluorescence was visualized by fluorescence microscopy (Zeiss Axiostar Plus fluorescence microscope) using the following filters: excitation 470/40 nm, emission 525/50 nm (FITC).

Animal Tumor Model. Animal model of human ovarian carcinoma xenografts was used as previously described (Dharap et al., 2005; Minko et al., 2000). Briefly A2780 human ovarian cancer cells (2×10^6) were subcutaneously transplanted into the flanks of female athymic nu/nu mice. When the tumors reached a size of about 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 (Dharap et al., 2005; Minko et al., 2000). Animal weight was measured every day within one week after the treatment.

Antitumor activity. Changes in tumor size were used as an overall marker for antitumor activity as previously described (Dharap et al., 2005; Minko et al., 2000). 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 oligo-nucleosomes) in homogenates of the tumor as previously described (Minko et al., 2000; Minko et al., 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 (Roche, Nutley, NJ) using terminal deoxynucleotidyl transferase mediated dUTP-fluorescein nick end labeling (TUNEL) method as previously described (Dharap et al., 2005; Pakunlu et al., 2004). Apoptosis induction in tumor was measured 24 hours 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, caspases 3 (*CASP3*), caspase 9 (*CASP9*). as previously described (Dharap and Minko, 2003; Pakunlu et al., 2003; Pakunlu et al., 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 TTG TGG CCT TCT TTG AG (sense), CCA AAC

TGA GCA GAG TCT TC (antisense); *BCLXL* – ATG AAC TCT TCC 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 AAG AT (antisense) β_2-*m* – ACC CCC ACT GAA AAA 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 1xTBE buffer (0.089 M Tris/Borate, 0.002 M ethylenediaminetetraacetic acid, EDTA, 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 RT-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 (ANOVA) and presented as mean value ± standard deviation (SD) from four to eight independent measurements in separate experiments.

JPET Fast Forward. Published on November 15, 2005 as DOI: 10.1124/jpet.105.094243
This article has not been copyedited and formatted. The final version may differ from this version.

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 fluorescein isothiocyanate (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 substantial higher fluorescence inside cancer cells when compared with non-conjugated 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 (Dharap et al., 2003; Holinger et al., 1999; Schimmer et al., 2001; Walsh et al., 2002).

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 can be seen from Fig. 3, bar 1, PEG-BH3 conjugate is relatively low in toxicity (high IC_{50} dose). Data show that conjugation of CPT with PEG polymer significantly ($P < 0.05$) increased its cytotoxicity when 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 when 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 equivalent dose of saline, free BH3, PEG or PEG-BH3 conjugate. Tumor size was measured 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 was 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 ($P < 0.05$) when compared to 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 homogenated 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. 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 all of 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 oligo-nucleosomes) 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, 5) and more pronouncedly with CPT-PEG conjugate (Fig. 6, Bar 6), 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 which includes an anticancer drug (camptothecin) as an apoptosis inducer, poly(ethylene glycol) polymer as a carrier and BCL2 homology 3 domain (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, 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 the literature (Gross et al., 1999; Kassim et al., 1999; Lutz, 2000; Mano et al., 1999; Reed, 1999; 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 mitochondrion to the cytoplasm leading to the activation of caspases-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; Lowe and Lin, 2000; Lutz, 2000; Reed, 1999). But 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; Lutz, 2000; Reed, 1999). 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, 2004; Minko et al., 1998). 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 (EPR) 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, when 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, being insoluble in water, required 10% of DMSO in cell culture media or PBS 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 (Rahier et al., 2005; Slichenmyer and Von Hoff, 1990; 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 (Greenwald et al., 2003; Minko et al., 2002a). 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 when 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 thean 40,000 times and apoptosis induction *in vivo* – more then 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. 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 when 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. 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 suppression of cellular antiapoptotic defense by BH3 peptide.

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 when compared with normal non-cancer 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 non-tumor 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 LHRH peptide for specific targeting of

drug delivery system to tumor cells (Dharap and Minko, 2003; Dharap et al., 2003; Dharap et al., 2005). We expect that such 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

NCHS (2004) *Health, United States, 2004*. National Center for Health Statistics, Hyattsville, Maryland.

Adams JM and Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. *Science* **281**:1322-1326.

Baekelandt M, Kristensen GB, Nesland JM, Trope CG and Holm R (1999) Clinical significance of apoptosis-related factors p53, Mdm2, and Bcl-2 in advanced ovarian cancer. *J Clin Oncol* **17**:2061-2068.

Conover CD, Pendri A, Lee C, Gilbert CW, Shum KL and Greenwald RB (1997) Camptothecin delivery systems: the antitumor activity of a camptothecin-20-0-polyethylene glycol ester transport form. *Anticancer Res* **17**:3361-3368.

Cosulich SC, Worrall V, Hedge PJ, Green S and Clarke PR (1997) Regulation of apoptosis by BH3 domains in a cell-free system. *Curr Biol* **7**:913-920.

Dharap SS and Minko T (2003) Targeted proapoptotic LHRH-BH3 peptide. *Pharm Res* **20**:889-896.

Dharap SS, Qiu B, Williams GC, Sinko P, Stein S and Minko T (2003) Molecular targeting of drug delivery systems to ovarian cancer by BH3 and LHRH peptides. *J Control Release* **91**:61-73.

Dharap SS, Wang Y, Chandna P, Khandare JJ, Qiu B, Gunaseelan S, Stein S, Farmanfarmaian A and Minko T (2005) Tumor-specific targeting of an anticancer drug delivery system by LHRH peptide. *Proc Natl Acad Sci U S A* **102**:12962-12967.

Fennelly D (1995) Dose intensity in advanced ovarian cancer: have we answered the question? *Clin Cancer Res* **1**:575-582.

Greenwald RB (2001) PEG drugs: an overview. *J Control Release* **74**:159-171.

Greenwald RB, Zhao H and Xia J (2003) Tripartate poly(ethylene glycol) prodrugs of the open lactone form of camptothecin. *Bioorg Med Chem* **11**:2635-639.

Gross A, McDonnell JM and Korsmeyer SJ (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* **13**:1899-1911.

Herod JJ, Eliopoulos AG, Warwick J, Niedobitek G, Young LS and Kerr DJ (1996) The prognostic significance of Bcl-2 and p53 expression in ovarian carcinoma. *Cancer Res* **56**:2178-2184.

Holinger EP, Chittenden T and Lutz RJ (1999) Bak BH3 peptides antagonize Bcl-xL function and induce apoptosis through cytochrome c-independent activation of caspases. *J Biol Chem* **274**:13298-13304.

Johnson AL (1999) Mcl-1--just another antiapoptotic Bcl-2 Homolog? *Endocrinology* **140**:5465-5468.

Kassim SK, Ali HS, Sallam MM, Fayed ST, Seada LS, abd-Elkawy E, Seada MA and Khalifa A (1999) Increased bcl-2 expression is associated with primary resistance to chemotherapy in human epithelial ovarian cancer. *Clin Biochem* **32**:333-338.

Lowe SW and Lin AW (2000) Apoptosis in cancer. *Carcinogenesis* **21**:485-495.

Lutz RJ (2000) Role of the BH3 (Bcl-2 homology 3) domain in the regulation of apoptosis and Bcl-2-related proteins. *Biochem Soc Trans* **28**:51-56.

Maeda H (2001) The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. *Adv Enzyme Regul* **41**:189-207.

Mano Y, Kikuchi Y, Yamamoto K, Kita T, Hirata J, Tode T, Ishii K and Nagata I (1999) Bcl-2 as a predictor of chemosensitivity and prognosis in primary epithelial ovarian cancer. *Eur J Cancer* **35**:1214-1219.

Minko T, Dharap SS, Pakunlu RI and Wang Y (2004) Molecular targeting of drug delivery systems to cancer. *Curr Drug Targets* **5**:389-406.

Minko T, Kopeckova P and Kopecek J (1999) Chronic exposure to HPMA copolymer-bound adriamycin does not induce multidrug resistance in a human ovarian carcinoma cell line. *J Control Release* **59**:133-148.

Minko T, Kopeckova P and Kopecek J (2000) Efficacy of the chemotherapeutic action of HPMA copolymer-bound doxorubicin in a solid tumor model of ovarian carcinoma. *Int J Cancer* **86**:108-117.

Minko T, Kopeckova P, Pozharov V and Kopecek J (1998) HPMA copolymer bound adriamycin overcomes MDR1 gene encoded resistance in a human ovarian carcinoma cell line. *J Control Release* **54**:223-233.

Minko T, Paranjpe PV, Qiu B, Lalloo A, Won R, Stein S and Sinko PJ (2002a) Enhancing the anticancer efficacy of camptothecin using biotinylated poly(ethylene glycol) conjugates in sensitive and multidrug-resistant human ovarian carcinoma cells. *Cancer Chemother Pharmacol* **50**:143-150.

Minko T, Stefanov A and Pozharov V (2002b) Selected contribution: Lung hypoxia: antioxidant and antiapoptotic effects of liposomal alpha-tocopherol. *J Appl Physiol* **93**:1550-1560; discussion 1549.

Oltvai ZN, Milliman CL and Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**:609-619.

Pakunlu RI, Cook TJ and Minko T (2003) Simultaneous modulation of multidrug resistance and antiapoptotic cellular defense by MDR1 and BCL-2 targeted antisense oligonucleotides enhances the anticancer efficacy of doxorubicin. *Pharm Res* **20**:351-359.

Pakunlu RI, Wang Y, Tsao W, Pozharov V, Cook TJ and Minko T (2004) Enhancement of the efficacy of chemotherapy for lung cancer by simultaneous suppression of multidrug resistance and antiapoptotic cellular defense: novel multicomponent delivery system. *Cancer Res* **64**:6214-6224.

Rahier NJ, Cheng K, Gao R, Eisenhauer BM and Hecht SM (2005) Synthesis of 14-azacamptothecin, a water-soluble topoisomerase I poison. *Org Lett* **7**:835-837.

Reed JC (1999) Dysregulation of apoptosis in cancer. *J Clin Oncol* **17**:2941-2453.

Schimmer AD, Hedley DW, Chow S, Pham NA, Chakrabartty A, Bouchard D, Mak TW, Trus MR and Minden MD (2001) The BH3 domain of BAD fused to the Antennapedia peptide induces apoptosis via its alpha helical structure and independent of Bcl-2. *Cell Death Differ* **8**:725-733.

Schuyer M, van der Burg ME, Henzen-Logmans SC, Fieret JH, Klijn JG, Look MP, Foekens JA, Stoter G and Berns EM (2001) Reduced expression of BAX is associated with poor prognosis in patients with epithelial ovarian cancer: a multifactorial analysis of TP53, p21, BAX and BCL-2. *Br J Cancer* **85**:1359-1367.

Searcey M and Patterson LH (2004) Resistance in cancer: a target for drug discovery. *Curr Med Chem Anti-Canc Agents* **4**:457-460.

Slichenmyer WJ and Von Hoff DD (1990) New natural products in cancer chemotherapy. *J Clin Pharmacol* **30**:770-788.

Srivastava V, Negi AS, Kumar JK, Gupta MM and Khanuja SP (2005) Plant-based anticancer molecules: A chemical and biological profile of some important leads. *Bioorg Med Chem* **13**:5892-5908.

Tsuruo T, Naito M, Tomida A, Fujita N, Mashima T, Sakamoto H and Haga N (2003) Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal. *Cancer Sci* **94**:15-21.

Walsh M, Lutz RJ, Cotter TG and O'Connor R (2002) Erythrocyte survival is promoted by plasma and suppressed by a Bak-derived BH3 peptide that interacts with membrane-associated Bcl-X(L). *Blood* **99**:3439-3448.

Zhang Z, Tanabe K, Hatta H and Nishimoto S (2005) Bioreduction activated prodrugs of camptothecin: molecular design, synthesis, activation mechanism and hypoxia selective cytotoxicity. *Org Biomol Chem* **3**:1905-1910.

Footnotes

This research is supported by NIH grant CA 100098 from the National Cancer Institute.

Reprint requests:

Tamara Minko, Ph.D.

Department of Pharmaceutics

Ernest Mario School of Pharmacy

Rutgers, The State University of New Jersey

160 Frelinghuysen Road

Piscataway, NJ 08854–8020

Phone: 732-445-3831 x 214

Fax: 732-445-3134

Email: minko@rci.rutgers.edu

Legends for figures

Fig. 1. Sequence of BCL2 homology 3 domain (BH3) peptide and structures of camptothecin (CPT) and CPT-PEG-BH3 conjugate. The conjugation site in CPT (hydroxyl at C20 position) is highlighted.

Fig. 2. Cellular internalization of BH3 peptide and PEG-BH3 conjugate in A2780 human ovarian carcinoma cells. BH3 peptide was labeled with fluorescein isothiocyanate (FITC) and fluorescence was registered by a fluorescent microscopy. The figure represents typical fluorescent microscopy images.

Fig. 3. Cytotoxicity of PEG-BH3 (1), CPT (2), CPT-PEG (3) and CPT-PEG-BH3 (4) in A2780 human ovarian carcinoma cells. IC_{50} doses were measured using a modified MTT assay. Cells were incubated 24 h with 45 different concentrations of active components. PEG and BH3 alone were not toxic in the concentrations used. Means ± SD from 8 independent measurements are shown.

$^{*}P < 0.05$ when compared with PEG-BH3

$^{\dagger}P < 0.05$ when compared with CPT

$^{\ddagger}P < 0.05$ when compared with CPT-PEG

Fig. 4. Time-course of growth of human ovarian tumor xenografts in mice as depicted by relative tumor size recorded from 6 to 96 h after treatment with: saline (control, 1), BH3 (2), PEG (3), PEG-BH3 (4), CPT (5), CPT-PEG (6), and CPT-PEG-BH3 (7). Means ± SD from 4-5 independent measurements are shown.

* $P < 0.05$ when compared with tumor treated with saline

$^{+}$ $P < 0.05$ when compared with tumor treated with CPT

‡ $P < 0.05$ when compared with tumor treated with CPT-PEG

Fig. 5. Effect of treatment with saline (1), BH3 (2), PEG (3), PEG-BH3 (4), CPT (5), CPT-PEG (6), and CPT-PEG-BH3 (7) on the expression of the genes encoding BCL2 (*BCL2*) and BCLXL (*BCLXL*) proteins, caspases 9 (*CASP9*) and 3 (*CASP3*) in tumor of mice bearing xenografts of A2780 human ovarian carcinoma. Means ± SD from 4-8 independent measurements are shown.

* $P < 0.05$ when compared with untreated tumor

$^{+}$ $P < 0.05$ when compared with tumor treated with CPT

† $P < 0.05$ when compared with tumor treated with CPT-PEG

Fig. 6. Apoptosis induction in tumor after treatment with saline (control, 1), BH3 (2), PEG (3), PEG-BH3 (4), CPT (5); CPT-PEG (6) and CPT-PEG-BH3 (7) conjugates of mice bearing xenografts of A2780 human ovarian carcinoma. Apoptosis was analyzed 24 h after the treatment by the measurement of the enrichment of histone-associated DNA

fragments (mono- and oligo-nucleosomes) in homogenates of the tumor. The enrichment in control cells was set to 1 unit, and the degree of apoptosis was expressed in relative units.

* $P < 0.05$ when compared with untreated tumor

$^{+}$ $P < 0.05$ when compared with tumor treated with CPT

† $P < 0.05$ when compared with tumor treated with CPT-PEG

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.

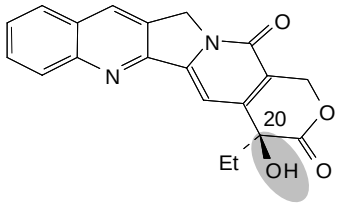
Fig. 8. Schematic illustration of cell death induction by traditional chemotherapy and proposed proapoptotic anticancer drug delivery system.

JPET Fast Forward. Published on November 15, 2005 as DOI: 10.1124/jpet.105.094243
This article has not been copyedited and formatted. The final version may differ from this version.

BH3

Ac–Met–Gly–Gln–Val–Gly–Arg–Gln–Leu–Ala–Ile–
Ile–Gly–Asp–Asp–Ile–Asn–Arg–Arg–Tyr–Cys–NH_2

CPT



Conjugation site in CPT ~ Hydroxyl at C20

CPT–PEG–BH3

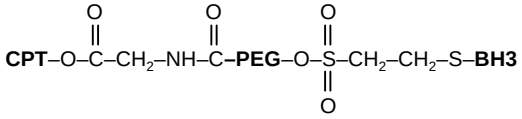


Fig. 1

JPET Fast Forward. Published on November 15, 2005 as DOI: 10.1124/jpet.105.094243
This article has not been copyedited and formatted. The final version may differ from this version.

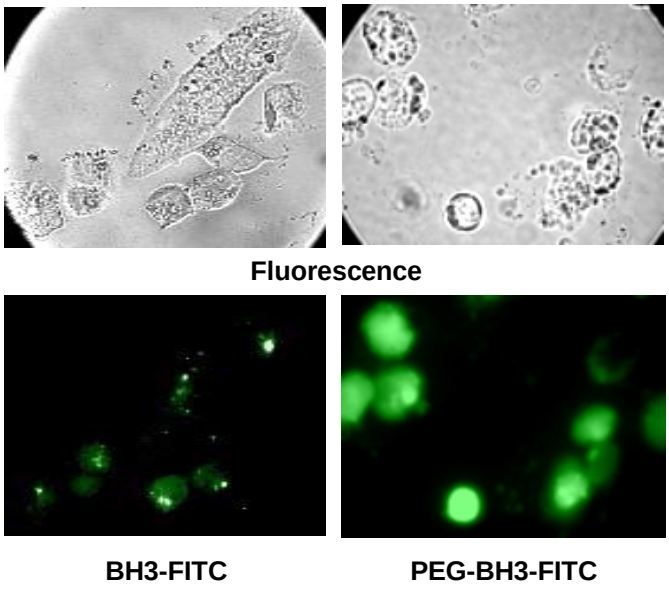


Fig. 2

JPET Fast Forward. Published on November 15, 2005 as DOI: 10.1124/jpet.105.094243
This article has not been copyedited and formatted. The final version may differ from this version.

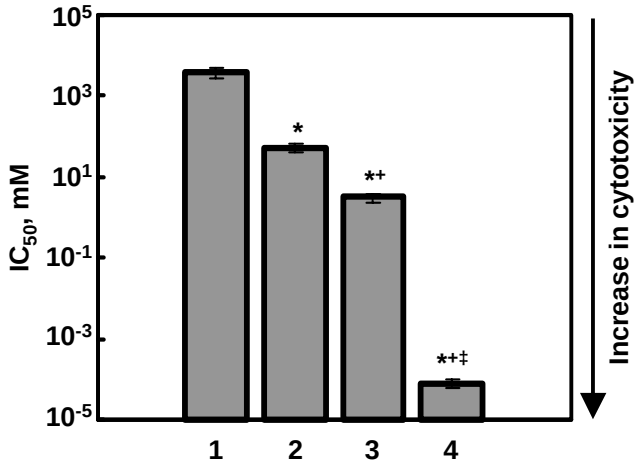


Fig. 3

JPET Fast Forward. Published on November 15, 2005 as DOI: 10.1124/jpet.105.094243
This article has not been copyedited and formatted. The final version may differ from this version.

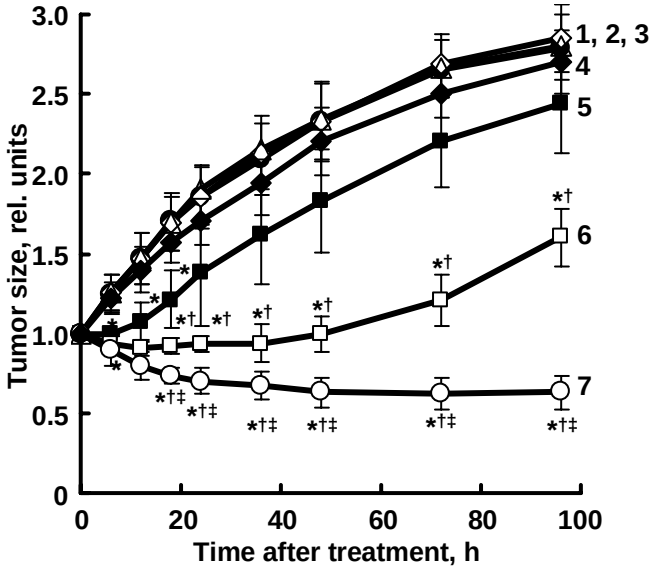


Fig. 4

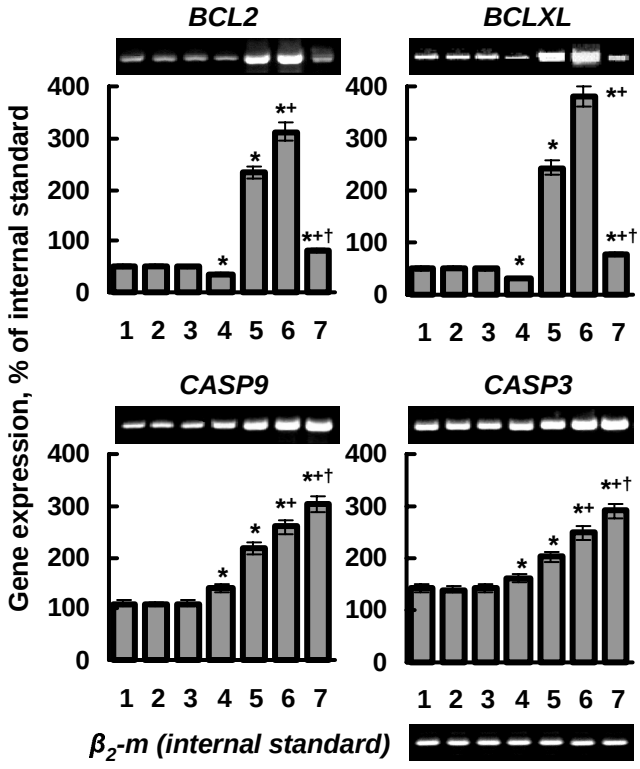


Fig. 5

JPET Fast Forward. Published on November 15, 2005 as DOI: 10.1124/jpet.105.094243
This article has not been copyedited and formatted. The final version may differ from this version.

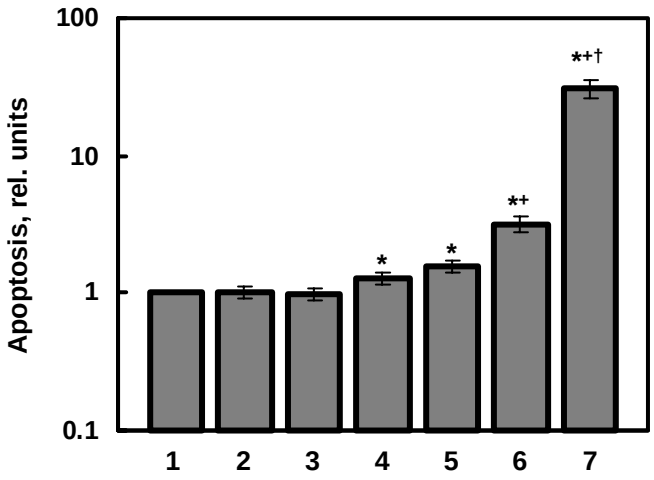


Fig. 6

JPET Fast Forward. Published on November 15, 2005 as DOI: 10.1124/jpet.105.094243
This article has not been copyedited and formatted. The final version may differ from this version.

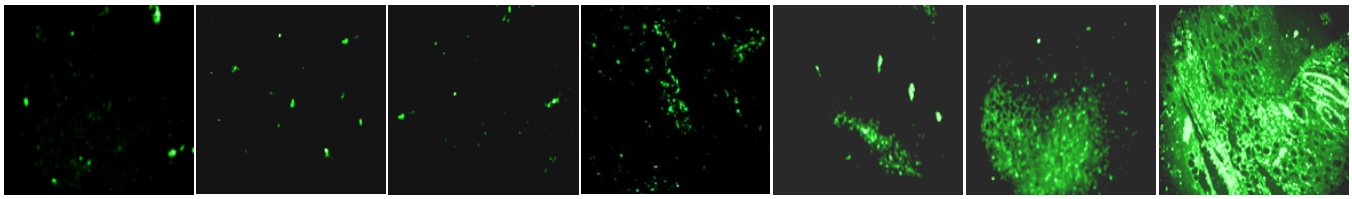


Fig. 7

JPET Fast Forward. Published on November 15, 2005 as DOI: 10.1124/jpet.105.094243
This article has not been copyedited and formatted. The final version may differ from this version.

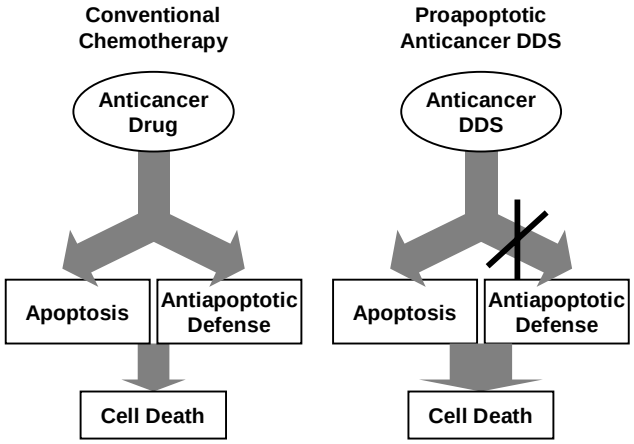


Fig. 8