Co-delivery of Cell-permeable Chimeric Apoptosis AVPIR$_8$ Peptide/p53 DNA for Cocktail Therapy

Huiyuan Wang, Qianqian Guo, Yifan Jiang, Ergang Liu, Yongxing Zhao, Huixin Wang, Yaping Li, and Yongzhuo Huang**

The tetra-peptide AVPI, derived from the Smac/DIABLO N-terminal epitope, is able to trigger caspase activation and apoptotic process. However, its clinical value is greatly hampered by the nature of membrane-impermeability. Herein, the cell-penetrating chimeric apoptotic peptide of AVPIR$_8$ is synthesized, of which the apoptosis-induced AVPI is strategically blended with the cell-penetrating sequence of octaarginine (R$_8$). The dual-functionalized AVPIR$_8$ is not only potent in inducing apoptosis in tumor cells due to the cell penetration ability, but also is able to work as gene carrier for transfering the tumor suppressor p53 DNA into cells, thus constructing a co-delivery drug system (AVPIR$_8$/p53). Such system efficiently promotes apoptosis in cancer cells while sparing normal cells, and its antitumor activity is further significantly enhanced in combination with doxorubicin as cocktail therapy. More importantly, the anticancer efficacy of the cocktail is demonstrated to be able to arrest tumor growth in two animal tumor models (melanoma and cervical cancers), respectively. The chemotherapeutic dose in the AVPIR$_8$/p53-based cocktail is significantly reduced by 80%, compared to the monotherapy of doxorubicin. The present results show the promise of the co-delivered AVPIR$_8$/p53 as adjuvant therapy for boosting the conventional chemotherapeutics, with a unique benefit of enhanced productive treatment outcomes yet greatly reduced adverse toxicity.

1. Introduction

Apoptosis is a process of programmed cell death that is crucial to maintain an organism's life cycle in health. Defective apoptosis occur in many diseases; for instance, abnormal inhibition of apoptosis is a hallmark of cancer,[1] in which the strictly controlled apoptotic process is suppressed in tumor cells, thus enabling them to escape the preset destiny of death and survive even in harsh environments such as hypoxia and malnutrition. Various pathways are involved in tumor cells for limiting or inhibiting apoptosis; the common ones are the overexpression of inhibitors of apoptosis proteins (IAPs) and the malfunction of P53 tumor suppressor.[2] Therefore, targeting these intrinsic apoptotic pathways by forcing cancer cells to commit suicide is an emerging anticancer therapy.[3] Additionally, to effectively induce apoptosis via activating IAPs and p53 is an important means for enhancing the sensitivity of tumor cells to chemotherapy.[4]

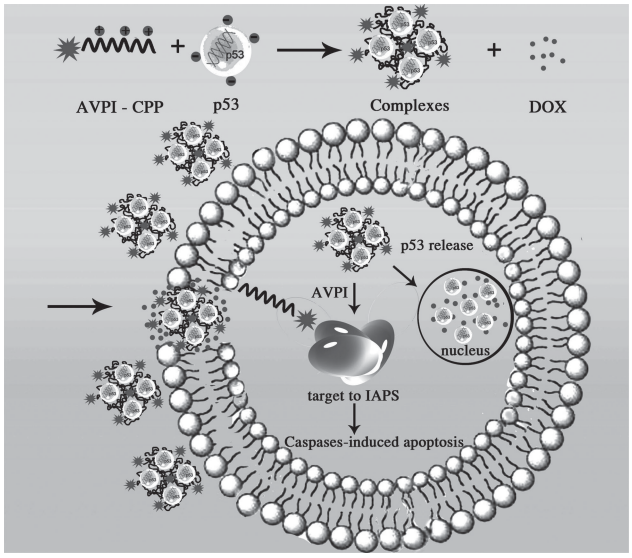
Mitochondrial release of the second mitochondrial-derived activator of caspase (Smac)/direct inhibitor of apoptosis protein-binding protein with low isoelectric point (pI) (DIABLO) dimer is a general feature of apoptosis.[5] The Smac/DIABLO translocate into the cytosol in response to apoptotic stimuli, and subsequently bind to inhibitor of IAPs, thus lifting their inhibitory effect on caspase activation and apoptotic process.[6]

Smac is a 239-amino-acid precursor. Its N-terminal sequence is the active domain for Smac function, and interestingly, a mutation of the first amino acid at N-terminus can completely deactivate the Smac.[7] The apoptosis induced by chemotherapeutics could be significantly enhanced by gene transfer of Smac into cancer cells, via a mechanism of procaspase-3 activation.[8,9] More specifically, a tetra-peptide of AVPI derived from the N-terminal sequence of Smac has high binding affinity to the X-linked IAP (XIAP), with a K_d value of 400 nm,[10] suggesting the therapeutic value of Smac AVPI. Despite of the potency at molecular level, however, AVPI did not display effect on tumor cells, owing to its cell-impermeable nature.[11]

Herein, we constructed a cell-permeable chimeric Smac peptide by fusion of Smac sequence (AVPI) with a cell-penetrating polyarginine peptide (R$_8$) for cancer therapy. More importantly, we hypothesized that such a chimeric Smac peptide not only functioned as a potent proapoptotic agent, but also served as a carrier for co-delivering the p53 tumor suppressor gene into tumor cells, thereby composing combined therapeutics. Cocktail is believed to be a promising treatment that targets multiple biological and pathological processes critical for cancer growth and metastasis.[12] Therefore, the feasibility of this drug co-delivery system (AVPIR$_8$/p53) as adjuvant therapy combined

Dr. H. Y. Wang, Q. Q. Guo, Y. F. Jiang, E. G. Liu, Prof. Y. P. Li, Prof. Y. Z. Huang
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
501 Hai-ke Rd, Shanghai, 201203, China
E-mail: ypli@simm.ac.cn; yzhuang@simm.ac.cn
Dr. H. X. Wang, Prof. Y. X. Zhao
Zhengzhou University School of Pharmaceutical Sciences
Zhengzhou, 450001, China
Prof. Y. Z. Huang
The Key Laboratory of Smart Drug Delivery (Fudan University)
MOE & PLA, Shanghai, 201203, China

DOI: 10.1002/adfm.201300793



Scheme 1. Co-delivery of cell-permeable chimeric Smac peptide/p53 gene into tumor cells as a cocktail strategy with doxorubicin for cancer therapy.

with doxorubicin (DOX) was also studied, and the cocktail strategy is illustrated as **Scheme 1**. Our goal was to develop an effective yet safe cocktail for cancer therapy.

2. Results

2.1. Characterization

The nanoscale and compact complex (vector/gene) is favorable for gene delivery. The particle sizes and zeta potentials of $AVPIR_8$/pDNA complexes at varying w/w ratios were measured. A typical trend was seen that with the increase of w/w ratio, the particle size of complexes gradually decreased (**Figure 1**A). Notably, a significant decline of size occurred when the w/w ratio rose from 20 to 30, suggesting the formation of compact nanostructure. By contrast, the zeta potential of complexes showed an increase trend with the elevating w/w ratio due to the surplus of cationic $AVPIR_8$. (Figure 2B) The zeta potential turned positive when the w/w ratio of $AVPIR_8$/pDNA was higher than 20. The occurrence of the turning point of zeta potential was in accordance with the change in particle sizes. Therefore, the ratio of $AVPIR_8$/pDNA was set at 30 for the in vitro and in vivo studies.

The formation of the compact complexes was further confirmed by the transmission electron microscopy (TEM) imaging (Figure 2C) and agarose gel electrophoresis (Figure 2D). Due to the vacuumed condition for measure, the size shown in TEM is smaller than the dynamic scattering light method (DSL) results. The electrophoretic mobility of DNA was completely retarded at the w/w ratio of 30, indicating the optimal ratio for the effective binding and stable complexes of $AVPIR_8$/pDNA.

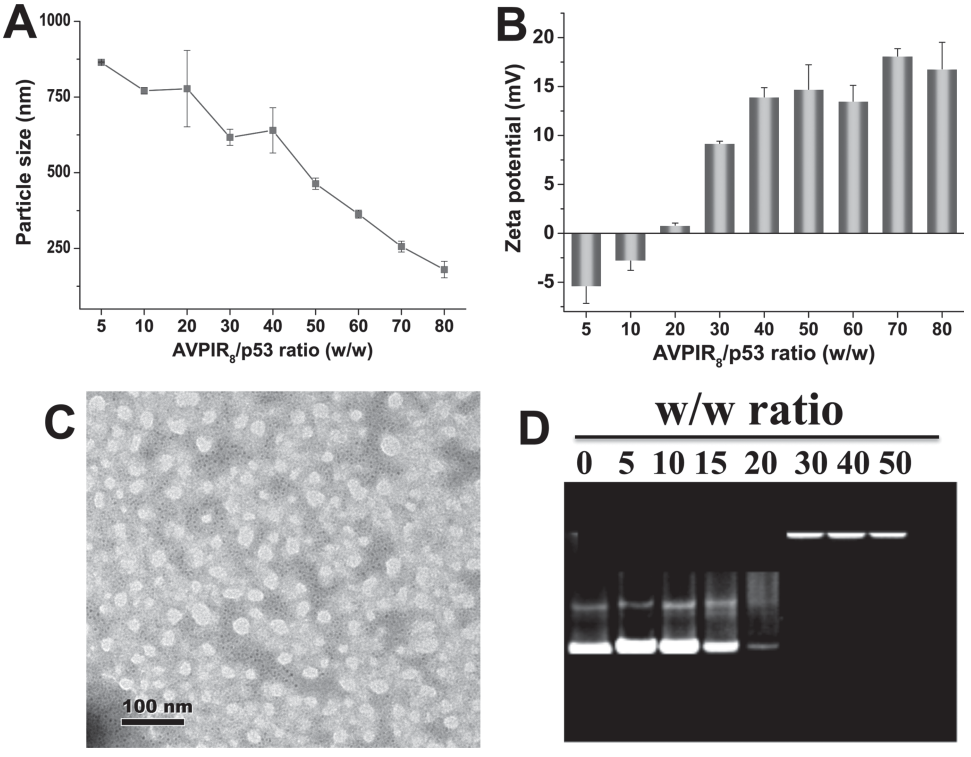


Figure 1. Characterization of the $AVPIR_8$/p53 DNA nano complexes. A) particle size measurement by DLS; B) zeta potential; C) TEM image; and D) Gel retardation assay.

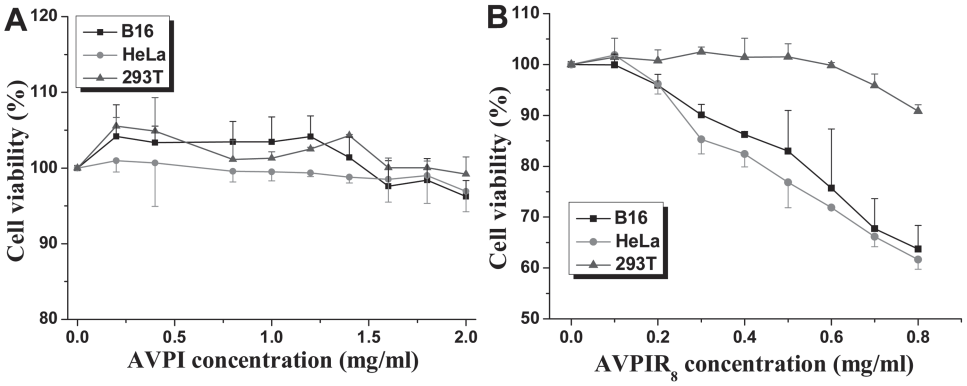


Figure 2. Cell growth inhibitory effect on A) the cell-impermeable AVPI and B) the cell-penetrating $AVPIR_8$.

2.2. In Vitro Cytotoxicity

The N-terminal tetra-peptide (AVPI) of the mature Smac protein has been demonstrated to be able to bind and inhibit XIAP.[14] Nevertheless, there lacks either active or passive mechanism for AVPI transporting across cell membrane, rendering it virtually no pharmacological effect on tumor cells. To overcome this problem, intracellular delivery assisted by CPP was employed. The cell-penetrating polyarginine peptide, such as R_8, has been widely used to bind and condense nucleic acid drugs via charge interaction, and shuttle the cargo genes into cells,[15] and it is highly biocompatible.[16] Moreover, polyarginine as a drug candidate for treating vein graft, has been investigated in either experimental animal models [17] or clinical trials (ClinicalTrials.gov Identifier: NCT00264706). Therefore, it is safe for biomedical application. We generated a chimeric Smac peptide via solid-phase synthesis with a sequence of $AVPIR_8$, in order to enhance the cellular uptake and transgene efficiency. To investigate the cell permeability of $AVPIR_8$, cytotoxicity studies were conducted in HeLa cells, B16 cells, and 293T cells. As expected, there was no obvious toxicity of AVPI observed in all cell lines, even at high concentrations up to 2 mg mL^{-1}, due to its cell-impermeable nature (**Figure 2**A). $AVPIR_8$, however, displayed a cytotoxic effect on tumor cells at a dose-dependent fashion at the test range. For example, the viability was shown 64% in B16 melanoma cells and 62% in HeLa cells at a concentration of 0.8 mg mL^{-1} of $AVPIR_8$ (**Figure** 3B). Interestingly, $AVPIR_8$ did not affect the proliferation of the non-tumoral 293T cell line, with cell viability greater than 95% at all the test concentrations. Therefore, the feature of selective action on tumor cells indicated the safety of $AVPIR_8$ for in vivo therapy.

2.3. In Vitro Transfection of $AVPIR_8$/pDNA

The optimal ratio of $AVPIR_8$/pDNA (30:1) was further confirmed by the in vitro transfection study. The luciferase expression efficiency was assessed in B16, HeLa, and 293T cells, using pGL-3 plasmid as a reporter gene. The best outcomes of gene transfection in all the test cell lines were achieved by using the $AVPIR_8$/pDNA ratio of 30 (**Figure 3**), and thus this ratio was used for the following studies.

2.4. Tumor Cell Growth Inhibition of the Cocktail

The antitumor efficacy was investigated for the cocktail (triple) therapy containing the apoptosis peptide, p53 tumor suppressor gene, and chemotherapeutic agent DOX. The individual use of the proapoptotic $AVPIR_8$ or p53 within the selected dose range showed the ability of growth inhibition, but the efficacy hardly met the treatment expectation, and neither did the single use of DOX at the test concentrations (up to 0.8 μg mL^{-1}) (**Figure 4**). However, the combined use of $AVPIR_8$ and DOX was significantly potent than the single use of DOX, and the $AVPIR_8$ remarkably boosted the DOX-directed toxic effect on tumor cells due to its apoptosis-induced function and the sensitization effect. Further, the triple therapy ($AVPIR_8$/p53+DOX) displayed remarkable synergetic effects and the strongest tumoricidal activity; the cell viability was measured to be respectively 35.8% (with low DOX dose) and 18.8% (with high DOX dose) in B16 cells, and 26.2% (with low DOX dose) and 13.5% (with high DOX dose) in HeLa cell line. The cocktail exhibited better outcomes than single use of DOX or any dual combination. Tumor cell viability with the cocktail therapy

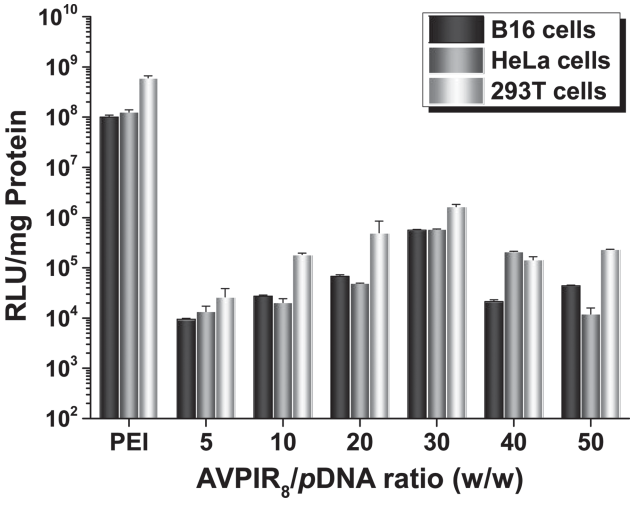


Figure 3. In vitro gene transfection mediated by $AVPIR_8$.

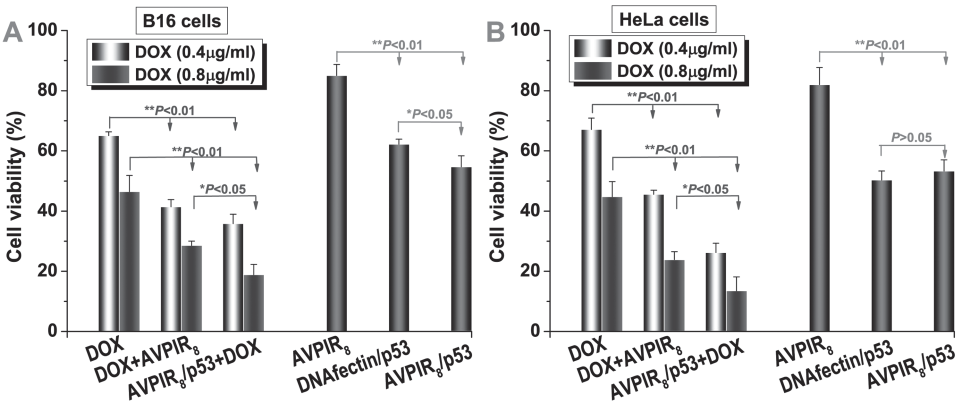


Figure 4. Anticancer activity of the cocktail on A) B16 cell line and B) HeLa cell line. The w/w ratio of the codelivery system of $AVPIR_8$/p53 was set 30:1, and the $AVPIR_8$ dose was 0.3 mg mL^{-1}, while the DOX dose was 0.4 or 0.8 μg mL^{-1}.

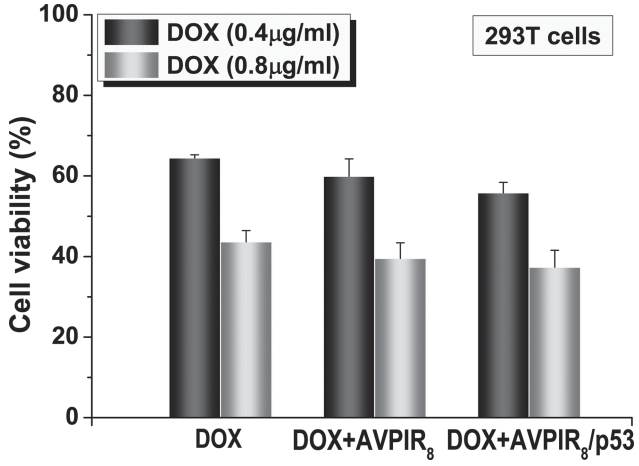


Figure 5. The apoptotic effect on non-tumoral 293T cells.

showed remarkably 2.5-fold lower than that of the single DOX (18.8% vs 46.5%) in B16 cells, and a 3.3-fold decrease in HeLa cells (13.5% vs 44.8%). Most importantly, the cocktail could greatly reduce the dose of chemotherapeutic agents. For instance, tumor cells treated with the cocktail containing DOX (0.4 μg mL^{-1}) showed significantly lower viability compared to those treated with high dose of DOX (0.8 μg mL^{-1}) alone, measuring 35.8% versus 46.5% in B16 cells, and 26.2% versus 44.8% in HeLa cells. These results demonstrated the unique benefit of greatly improved antitumor activity yet with half-reduced chemotherapeutic dose.

In contrast to the results from tumor cells, the cocktail containing 0.4 μg mL^{-1} of DOX showed significantly lower cytotoxicity than the high-dose DOX (0.8 μg mL^{-1}) in the non-tumoral 293T cell line (**Figure 5**). It suggested that the tumor cells were specifically sensitive to the cocktail compared to the normal cells, and this selective action pattern potentially provided advantage of reduced adverse effects on healthy cells. As evidence, the groups containing low-dose DOX (0.4 μg mL^{-1}) all displayed considerable cell viability (>55%). For those containing high-dose DOX (0.8 μg mL^{-1}), the cell viability still remained about 40% on non-tumoral 293T cells, and no significant difference in cytotoxicity was shown among the groups with or without AVPIR8 and p53. It suggested that DOX was the contributing factor to the toxicity on the non-tumoral cells, further demonstrating the safety of the codelivery system of $AVPIR_8$/p53. This could be accounted for the targeting effect on intrinsic apoptosis in tumor cells of the AVPI and p53 gene.

2.5. In Vivo Treatment Efficacy

The therapeutic efficacy of cocktail method was further evaluated by monitoring tumor growth in two separate animal tumor models (C57 mice bearing B16 melanoma and Balb/c-nu nude mice harboring HeLa cervical tumor). The cocktail-treated groups displayed the best therapeutic outcomes in C57 mouse model. The melanoma tumor sizes after treatment were 330 mm^3 in the cocktail group containing 0.5 mg kg^{-1} DOX, and 185 mm^3 in the cocktail group containing 1 mg kg^{-1} DOX (**Figure 6**A), with tumor inhibition rate of 78.4% and 90.2%, respectively (Figure 6B). By sharp contrast, the groups given DOX alone showed the inhibition rate of 27.1% (0.5-mg group) and 50.1% (1-mg group). The notable benefit of cocktail therapy is the greatly reduced dose of DOX. For instance, the cocktail containing DOX (0.5 mg kg^{-1}) surpassed the single use of high-dose DOX (1 mg kg^{-1}); the inhibition rate was 78.4% versus 50.1%. The findings agreed with the cellular results.

No significant difference in body weight changes was observed in the cocktail-treated groups during the experimental period (Figure 6C). It suggested that there was no identified severe adverse toxicity caused by the cocktail method, but the DOX (1 mg kg^{-1}) group experienced the obvious loss of body weight.

Furthermore, we investigated the efficacy of the cocktail method on the transplanted humanized tumor mice. Echoed with the results in C57 mice, the group treated by cocktail ($AVPIR_8$/p53+DOX) showed the strongest growth inhibition: the tumor size was measured to be 118 mm^3 at experimental endpoint. By contrast, the tumor sizes were 1391 mm^3 in the control PBS group, 149 mm^3 in the DOX group (2.5 mg kg^{-1} d^{-1}), 518 mm^3 in the low-dose DOX group (1 mg kg^{-1} $2d^{-1}$), 255 mm^3 in the [DOX+$AVPIR_8$] group (**Figure 7**A,B). The inhibition

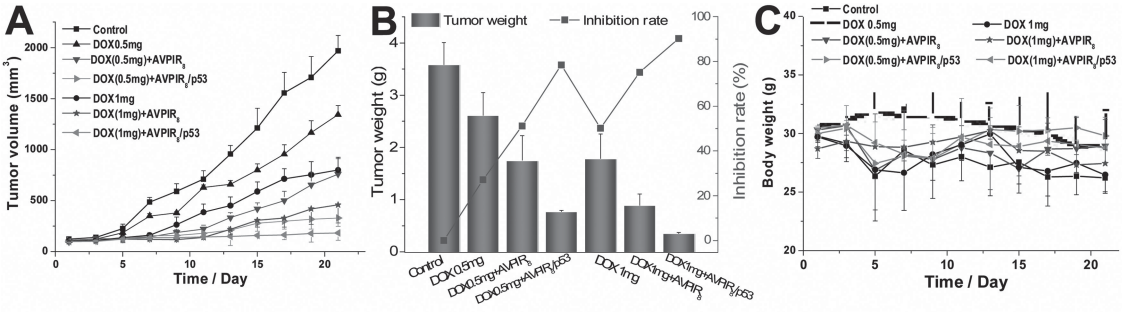


Figure 6. The in vivo antitumor efficacy of the cocktail in C57 mice bearing melanoma. A) The tumor volume over the treatment regimen, B) the tumor weight at the experimental endpoint and the inhibition rate of tumor growth, C) the change of the body weight over the regimen.

rate in the cocktail-treated group was 92.7%, surpassing the single use of DOX (inhibition rate of 48.9% and 91.1% for 1 mg kg^{-1} $2d^{-1}$ and 2.5 mg kg^{-1} d^{-1}, respectively). The dual combination therapy ($AVPIR_8$+DOX 1mg, inhibition rate of 81.6%) also exhibited better than the single use of DOX (1 mg) (Figure 7C). Although the single use of DOX (2.5 mg kg^{-1} d^{-1}) reached a remarkable inhibition rate almost as high as the cocktail containing DOX (1 mg kg^{-1} $2d^{-1}$), the DOX dose in the former group was five-fold higher than that in the latter group. In addition, the animals receiving DOX (2.5 mg kg^{-1} d^{-1}) alone were found suffering from debilitation and significant weight loss, and none survived at day 21 due to the severe side toxicity, whereas the cocktail-treated mice were not observed to have noticeable adverse effects and body weight change (Figure 7D).

3. Discussion

Despite advances in chemotherapy, the five-year survival rate of cancer still remains desperately low, due to the severe toxic side effects and multidrug resistance (MDR).[18] Apoptosis of cancer cells can be provoked by a variety of stimuli, including chemotherapeutic drugs, tumor suppressor gene, radiotherapy, and thermotherapy.[19] However, a recent investigation revealed a backfire of radiotherapy that radiation-induced apoptosis could activate a growth signaling cascade in tumor cells and thereby stimulate tumor repopulation.[20] Therefore, development of biomimic regulators that specifically target the apoptotic pathways would be preferred. In response to apoptotic stimuli, proapoptotic proteins, e.g. Smac, are released into the

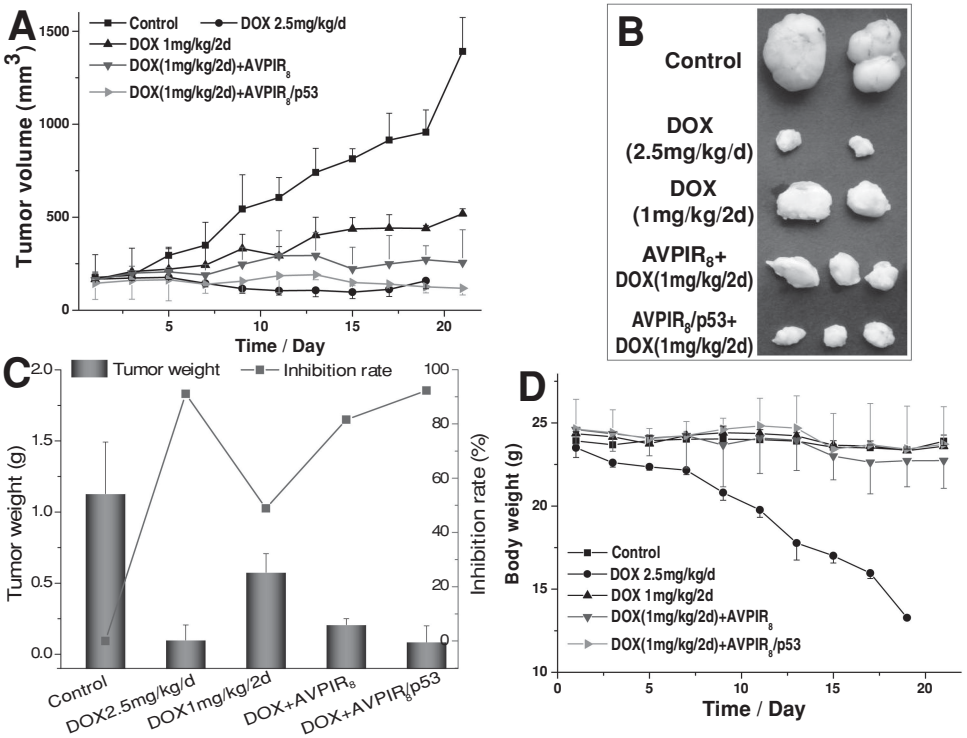


Figure 7. The in vivo antitumor efficacy of the cocktail in nude mice harboring HeLa cervical tumor. A) The tumor volume over the treatment regimen, B) the representative tumors after treatment, C) the tumor weight at the experimental endpoint and the inhibition rate of tumor growth, and D) the change of the body weight over the regimen.

cytoplasm from the mitochondria and thus trigger the intrinsic apoptotic pathway.[5] The polypeptide of Smac/DIABLO has been demonstrated to be able to enhance anticancer drug-induced apoptosis.[8,21] More specifically, the tetrapeptide AVPI from the Smac/DIABLO N-terminal epitope is able to interact with the XIAP-BIR3 domain, consequently inhibiting XIAP and sensitizing tumor cells to chemotherapeutics.[22] In this study, we developed a dual functionalized cell-penetrating chimeric apoptosis peptide of $AVPIR_8$, a fusion sequence of the shortest Smac N-terminal AVPI tetra-peptide and the cell-penetrating peptide of octaarginine (R_8). The AVPI is cell-impermeable and thus renders it ineffective in cancer therapy. Once fused with the R_8 cell-penetrating sequence, the AVPI becomes a potent proapoptotic agent.

Another important feature of the chimeric Smac $AVPIR_8$ for cancer therapy is its additional function as gene carrier, and the polycationic R_8 motif enables effective gene delivery. Tumor cells often evolve resistance to the intrinsic apoptosis mechanism. Using monotherapy is not sufficient to sensitize or induce apoptosis in tumor cells.[3] Therefore, the apoptosis regulator, p53 DNA, was selected as dual proapoptotic combination with $AVPIR_8$. The p53 can act through both extrinsic and intrinsic apoptotic signaling pathways by activation of the aspartate-specific caspases.[23] Because p53 is frequently mutated in more than 50% human cancers,[24] the reintroduction of p53 DNA can lead to tumor regression,[25] as well as the increased sensitivity of tumor cells to chemotherapeutic agents.[26] Our results demonstrated the better proapoptotic effect of the dual combination than any single use of AVPI or p53.

Cocktail, including various chemotherapeutics,[27] genes,[28] or combinations of chemical and nucleic acid drugs,[29] represents a promising strategy for curbing cancer development. The cocktail therapy (codelivery system of $AVPIR_8$/p53 combining DOX) displayed strong tumoricidal activity on tumor cells. In particular, the cocktail could greatly reduce the dose of the co-administered chemotherapeutic agent. It may attribute to the dual combination of proapoptotic agents, which synergistically initiate signal transduction cascade and activate multiple apoptotic pathways. More importantly, $AVPIR_8$/p53 did not exhibit adverse effect on non-tumoral cells, indicating normal cells are insensitive to the exogenous apoptotic regulators and thus can be spared during a hostile chemotherapeutic-involved regime.

In the B16 melanoma animal model, the cocktail effectively arrested the tumor growth, whereas the mice given the single DOX with the same dose suffered rapid tumor expansion. Although peritumoral administration employed in animal studies may not reflect the real-time profile of pharmacokinetics, it should be mentioned that because of the nanosize of the CPP/gene complex, CPP-mediated systemic delivery of nucleic acid drugs can accumulate in tumor via EPR effect.[30] Besides, local therapy represents a growing trend for its unique benefit of maximizing local tumor control with minimal systemic toxicity.[31] Malignant melanoma is one of the most aggressive skin cancers with active metastatic potential and high resistance to chemotherapeutics,[32] and conventional chemotherapy generally offers unproductive treatment outcomes. The findings reveal the feasibility of the potent combination for combating cancer, and thus are meaningful even in an early stage.

The tumor growth curves in nude mice bearing HeLa cervical tumor of the cocktail ($AVPIR_8$/p53 + 1 mg kg^{-1} DOX, q.o.d. dosing) and the single DOX (2.5 mg kg^{-1} q.d. dosing) were almost overlapped, and their tumor suppression rates were both greater than 90%. However, the high dose of DOX not only contributed to the arrested tumor growth, but also accounted for the 100% mortality rate of the treated animals, indicating its fatal adverse toxicity and the extremely narrow therapeutic window. By contrast, none died in the group receiving the cocktail therapy, benefiting from the sharply reduced chemotherapeutic dose by 80%.

4. Conclusions

Growing awareness is that monotherapy is hardly sufficient to treat cancers, and thus combined pharmacotherapy (i.e., cocktail) represents a solution to improved therapeutic efficacy with reduced adverse effects. Apoptosis targeting therapy is a promising strategy for combating cancer. The chimeric $AVPIR_8$ not only acts as a cell-permeable apoptotic peptide, but also functions as a drug carrier for the p53 tumor suppressor gene. Such a proapoptotic codelivery system could specifically induce synergistic apoptosis in tumor cells, but sparing normal cells. The co-delivered $AVPIR_8$/p53 DNA remarkably boosted the antitumor effect of DOX, and this cocktail not only successfully arrested tumor growth in two animal models bearing melanoma and cervical tumor, respectively, but also displayed benefits of greatly reduced dose and improved safety. Therefore, this co-delivery system of $AVPIR_8$/p53 DNA is promising as adjuvant for chemotherapeutics-involved cocktails for cancer therapy.

5. Experimental Section

Materials: N-hydroxybenzotriazole (HOBt), 2-Chlorotrityl chloride resin (100–200 mesh, loading: 1.32 mmol g^{-1}), o-benzotriazol-N,N,N',N'-tetramethyluronium hexa fluorophosphate (HBTU), N-fluorenyl-9-methoxycarbonyl (FMOC) protected L-amino acids (FMOC-Arg(pbf)-OH, FMOC-Ala-OH, FMOC-Val-OH, FMOC-Pro-OH, FMOC-Ile-OH) were purchased from GL Biochem Ltd, and used as received. Diisopropylethylamine (DiEA), piperidine, trifluoroacetic acid (TFA), methylacrylic acid polyethylene glycol single armor ether ester, and N,N-dime-thylformamide (DMF), and dichloromethane (DCM) were purchased from Shanghai Reagent Chemical Co. (Shanghai, China). Triisopropylsilane (TIS), branched polyethylenimine (PEI, M_w 25 kDa) were obtained from Sigma-Aldrich (USA). DOX was a product from Huannan Company (China). The Endofree Plasmid Purification Kit was purchased from Tiangen Biotech Co. Ltd. (China). GelRed was purchased from Biotium (Hayward, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), penicilline-streptomycin, 0.25% trypsin-EDTA solution, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Invitrogen (USA). The Micro BCA protein assay kit was acquired from Beyotime Institute of Biotechnology (Haimen, China). All other reagents were of analytical grade.

Cell Culture and Animals: HeLa human cervix carcinoma cells, mouse B16 melanoma cells, and human embryonic kidney transformed 293T cells were incubated in DMEM containing 10% FBS and 1% antibiotics at 37 °C in humidified atmosphere with 5% CO2. Female C57 mice and BALB/c-nu nude mice (4–5 weeks old, 18–22 g) were housed under specific pathogen-free conditions. Animals possessed continuous access

to sterilized food pellets and distilled water, and a 12-h light/dark cycle. All of the animals were in quarantine for a week before treatment. The experimental procedures were approved by the Institutional Animal Care and Use Committee.

Synthesis of the Chimeric Smac Peptide (AVPIR$_8$): The chimeric Smac peptide (AVPIR$_8$) containing the first four N-terminal amino acids of Smac (AVPI) and the cell penetrating peptide (RRRRRRRR, R$_8$) was synthesized using the standard solid phase synthesis based on classical Fmoc/tert-Butyl chemistry.[13] The final product was confirmed by MALDI-TOF Mass assay.

Preparation of AVPIR$_8$/p53: DNA Complexes The complexes were prepared by mixing particular volume of AVPIR$_8$ solution with 5 μL of p53 DNA (1 μg in 40 mm Tris-HCl buffer solution). Subsequently, saline was added into the complexes to a total volume of 100 μL and vortexed for 10 s. The mixture was incubated at 37 °C for 30 min to form stable complexes.

Characterization by Agarose Gel Electrophoresis, TEM, Particle Size, and Zeta Potential: The stability of AVPIR$_8$/p53 DNA complexes at varying w/w ratios (5–50) was measured by agarose gel electrophoresis. The resultant complexes were electrophoresed on the 0.8 w/v% agarose gel containing GelRed. Naked DNA was used as control. A Vilber Lourmat UV-transiluminator was used for the visualization of DNA. The morphological examination of the AVPIR$_8$/pDNA complex with w/w ratio of 30 was performed using TEM operating at an acceleration voltage of 100 kV, after negative staining with sodium phosphotungstate solution (0.2 w/v%). The particle size and zeta potential of AVPIR$_8$/pDNA complexes were measured by Zeta Sizer N particle analyzer (Malvern, UK) via DSL. The AVPIR$_8$/pDNA complexes at w/w ratios from 5 to 80 were prepared as described above, and dilution with deionized water was made for the sample before determination.

In Vitro Cytotoxicity Assay of the Smac Peptide AVPIR$_8$: The cytotoxicity assay in vitro was measured in HeLa, B16 and 293T cell lines by MTT assay. Cells were seeded in 96-well plates at a density of 6000 cells per well in 100 μL DMEM containing 10% FBS and cultured for 24 h before test. Cells were treated with AVPIR8 for 48 h. MTT solution (20 μL, 5 mg mL^{-1}) was added to each well with further incubation for 4 h. The medium was then removed and 200 μL DMSO was added to dissolve the formazan crystals. The absorption was measured at 570 nm using a microplate reader (Thermo, USA). The cell viability was calculated according to the equation as follows:

$$\text{Cell viability}(\%) = \left(OD_{samples} - OD_{DMSO}\right) / \left(OD_{control} - OD_{DMSO}\right) \times 100$$

In Vitro Transfection Mediated by AVPIR$_8$: The optimal ratio for transfection was determined by luciferase expression experiments, and pGL-3 DNA was used as a reporter gene. Cells were seeded in 24-well plates at a density of 6×10^4 cells per well and cultured in the complete DMEM medium (10% FBS) at 37 °C. After incubation for 24 h, the medium was replaced by fresh complete DMEM (10% FBS) containing the complexes at a pDNA dose of 1 μg per well. After incubation for 4 h the medium was replaced by fresh complete DMEM (10% FBS), and the cells were cultured for another 44 h. To assess the luciferase expression of pDNA, the cells were thoroughly washed, and then lysed with the reporter lysis buffer. Luciferase activity was determined with a chemiluminometer (Lumat LB9507, EG&G Berthold, Germany), and the total protein measured by a standard BCA method. Luciferase activity was denoted as RLU per mg proteins.

Cytotoxicity of the Cocktail Containing AVPIR8/p53 and Doxorubicin: Cell growth inhibition of the cocktail was evaluated in HeLa, B16, and 293T cells using the standard MTT assay according to the preceding procedure. Briefly, cells were seeded in 24-well plates and cultured for 24 h before use. In cocktail group, AVPIR8/p53 complexes at optimal ratio (w/w = 30) were added into each well with a final concentration of 300 μg/mL for AVPIR8 and 10 μg mL^{-1} for p53 DNA, and incubated for 4 h. Then the medium was subsequently replaced with 1 mL of fresh DMEM containing 0.4 or 0.8 μg DOX. After further culture for 44 h, 100 μL of MTT (5 mg mL^{-1}) solution was added into each well. Following 4 h of incubation, the medium was removed and DMSO was added to dissolve the formazan crystals. The absorption was measured as described above.

In Vivo Anti-Tumor Study: To establish the B16 tumor model, B16 cells (5×106 cells/mouse) were subcutaneously injected into the back of the C57 mice with a 25-gauge needle. The developed tumor was monitored every other day using calipers and tumor volume was calculated using the following formula:

$$V = W^2 \times L/2$$

where W and L respectively are the shortest and longest diameters. When tumors reached an average volume of about 100 mm^3, the treatment regimens were carried out by peritumoral administration with alternate-day (q.o.d.) dosing. The mice were randomized into 7 groups (6 mice per group). Group 1, PBS (control); Group 2, DOX (0.5 mg kg^{-1}); Group 3, DOX (0.5 mg kg^{-1}) and AVPIR$_8$ (0.6 mg); Group 4, DOX (0.5 mg kg^{-1}) and AVPIR$_8$ (0.6 mg)/p53 (20 μg); Group 5, DOX (1 mg kg^{-1}); Group 6, DOX (1 mg kg^{-1}) and AVPIR$_8$ (0.6 mg); Group 7, DOX (1 mg kg^{-1}) and AVPIR$_8$ (0.6 mg)/p53 (20 μg). The body weight of the mice and tumor size were measured daily. At day 21, the mice were killed, and their tumors were immediately harvested and weighed. The inhibition rate of tumor growth was calculated as follows:

$$\text{Inhibition rate}(\%) = \left[\text{tumor weight(treated)} / \text{tumor weight(control)}\right] \times 100$$

To further study the therapeutic effect of cocktail treatment for human cancer cells in vivo, another tumor model (BALB/c-nu nude mice harboring HeLa cervical tumor) was established. Briefly, the nude mice were received subcutaneous injection with 0.2 mL of HeLa cell suspension (1×10^8 cells) in the back. When the volume of the tumor xenograft reached about 100 mm^3, the mice were treated by peritumoral administration with q.o.d dosing for a 21-day regimen, whereas the positive control group was given DOX (2.5 mg kg^{-1}) with q.d. dosing. Experimental groups were as follows (6 mice per group). Group 1, PBS (control); Group 2, DOX (2.5 mg kg^{-1} d^{-1}); Group 3, DOX (1 mg kg^{-1}); Group 4, DOX (1 mg kg^{-1}) and AVPIR$_8$ (0.6 mg); Group 5, DOX (1 mg kg^{-1}) and AVPIR8 (0.6 mg)/p53 (20 μg). At the endpoint of the experiment, the mice were killed, and the tumors were immediately harvested and weighed.

Statistical Analysis: The quantitative data were expressed as mean ± S.D. Statistical significance was analyzed by Student's t-test. Statistical significance was inferred at a value of $P < 0.05$.

Acknowledgements

The authors thank the support from National Basic Research Program of China (973 Program 2013CB932503), and NSFC, China (91029743, 81172996). This work was also supported in part by Shanghai Pu-jiang Scholar Program (11PJ1411800) and the Chinese Postdoctoral Science Foundation (2012M510097, 2013T60478), as well as School of Pharmacy, Fudan University & The Open Project Program of Key Lab of Smart Drug Delivery (Fudan University), MOE & PLA, China (SDD2011–02).

Received: March 4, 2013
Revised: June 18, 2013
Published online: July 24, 2013

[1] D. Hanahan, R. A. Weinberg, *Cell* **2011**, *144*, 646.
[2] a) A. D. Schimmer, *Cancer Res.* **2004**, *64*, 7183; b) F. Chen, W. Wang, W. S. El-Deiry, *Biochem. Pharmacol.* **2010**, *80*, 724.
[3] C. Vangestel, C. Van de Wiele, G. Mees, M. Peeters, *Cancer Biother. Radiopharm.* **2009**, *24*, 395.

[4] a) Q. S. Tong, L. D. Zheng, L. Wang, F. Q. Zeng, F. M. Chen, J. H. Dong, G. C. Lu, *Cancer Gene. Ther.* **2005**, *12*, 509; b) C. Lu, W. S. El-Deiry, *Apoptosis* **2009**, *14*, 597.
[5] E. N. Shiozaki, Y. Shi, *Trends Biochem. Sci.* **2004**, *29*, 486.
[6] a) C. Adrain, E. M. Creagh, S. J. Martin, *EMBO J.* **2001**, *20*, 6627; b) C. Du, M. Fang, Y. Li, L. Li, X. Wang, *Cell* **2000**, *102*, 33.
[7] J. Chai, C. Du, J. W. Wu, S. Kyin, X. Wang, Y. Shi, *Nature* **2000**, *406*, 855.
[8] S. Fulda, W. Wick, M. Weller, K. M. Debatin, *Nat. Med.* **2002**, *8*, 808.
[9] Z. Pei, L. Chu, W. Zou, Z. Zhang, S. Qiu, R. Qi, J. Gu, C. Qian, X. Liu, *Hepatology* **2004**, *39*, 1371.
[10] S. Wang, *Curr. Top. Microbiol. Immunol.* **2011**, *348*, 89.
[11] S. Wang, L. Bai, J. Lu, L. Liu, C. Y. Yang, H. Sun, *J. Mammary Gland. Biol. Neoplasia.* **2012**, *17*, 217.
[12] O. Taratula, O. B. Garbuzenko, A. M. Chen, T. Minko, *J. Drug Target* **2011**, *19*, 900.
[13] a) G. B. Fields, R. L. Noble, *Int J Pept Protein Res* **1990**, *35*, 161; b) N. M. Moore, T. R. Barbour, S. E. Sakiyama-Elbert, *Mol. Pharm.* **2008**, *5*, 140.
[14] Z. Liu, C. Sun, E. T. Olejniczak, R. P. Meadows, S. F. Betz, T. Oost, J. Herrmann, J. C. Wu, S. W. Fesik, *Nature* **2000**, *408*, 1004.
[15] I. Nakase, T. Takeuchi, G. Tanaka, S. Futaki, *Adv. Drug Delivery Rev.* **2008**, *60*, 598.
[16] N. A. Alhakamy, C. J. Berkland, *Mol. Pharm.* **2013**, *10*, 1940.
[17] M. H. Kown, A. Yamaguchi, C. L. Jahncke, D. Miniati, S. Murata, J. Grunenfelder, M. L. Koransky, J. B. Rothbard, R. C. Robbins, *J. Thorac. Cardiovasc. Surg.* **2001**, *121*, 971.
[18] a) R. Siegel, C. DeSantis, K. Virgo, K. Stein, A. Mariotto, T. Smith, D. Cooper, T. Gansler, C. Lerro, S. Fedewa, C. Lin, C. Leach, R. S. Cannady, H. Cho, S. Scoppa, M. Hachey, R. Kirch, A. Jemal, E. Ward, *CA-Cancer J. Clin.* **2012**, *62*, 220; b) M. Tanaka, T. Okazaki, H. Suzuki, J. L. Abbruzzese, D. Li, *Cancer* **2011**, *117*, 744.
[19] a) J. C. Reed, *J. Clin. Oncol.* **1999**, *17*, 2941; b) M. Johannsen, B. Thiesen, U. Gneveckow, K. Taymoorian, N. Waldofner, R. Scholz, S. Deger, K. Jung, S. A. Loening, A. Jordan, *Prostate* **2006**, *66*, 97.
[20] Q. Huang, F. Li, X. Liu, W. Li, W. Shi, F. F. Liu, B. O'Sullivan, Z. He, Y. Peng, A. C. Tan, L. Zhou, J. Shen, G. Han, X. J. Wang, J. Thorburn, A. Thorburn, A. Jimeno, D. Raben, J. S. Bedford, C. Y. Li, *Nat. Med.* **2011**, *17*, 860.
[21] a) C. R. Arnt, S. H. Kaufmann, *Cell Death Differ.* **2003**, *10*, 1118; b) F. Guo, R. Nimmanapalli, S. Paranawithana, S. Wittman, D. Griffin, P. Bali, E. O'Bryan, C. Fumero, H. G. Wang, K. Bhalla, *Blood* **2002**, *99*, 3419.
[22] T. L. de Souza, D. Sanches, R. B. Goncalves, S. S. R. Pita, P. G. Pascutti, M. L. Bianconi, F. C. de Almeida, J. L. Silva, A. C. de Oliveira, *Biophys. Chem.* **2010**, *152*, 99.
[23] S. Haupt, M. Berger, Z. Goldberg, Y. Haupt, *J. Cell Sci.* **2003**, *116*, 4077.
[24] A. Ventura, D. G. Kirsch, M. E. McLaughlin, D. A. Tuveson, J. Grimm, L. Lintault, J. Newman, E. E. Reczek, R. Weissleder, T. Jacks, *Nature* **2007**, *445*, 661.
[25] J. Sjostrom, J. Bergh, *BMJ* **2001**, *322*, 1538.
[26] T. Stoklsa, J. Golab, *Acta Biochim. Pol.* **2005**, *52*, 321.
[27] F. Li, M. Danquah, S. Singh, H. Wu, R. Mahato, *Drug Delivery Transl. Res.* **2011**, *1*, 420.
[28] B. Nie, Z. Shen, J. B. Wen, O. G. Wong, W. D. Hsueh, L. F. Huo, H. F. Kung, B. Jiang, M. C. Lin, *Mol. Cancer Ther.* **2008**, *7*, 2855.
[29] O. B. Garbuzenko, M. Saad, V. P. Pozharov, K. R. Reuhl, G. Mainelis, T. Minko, *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 10737.
[30] Y. S. Choi, J. Y. Lee, J. S. Suh, Y. M. Kwon, S. J. Lee, J. K. Chung, D. S. Lee, V. C. Yang, C. P. Chung, Y. J. Park, *Biomaterials* **2010**, *31*, 1429.
[31] R. De Souza, P. Zahedi, C. J. Allen, M. Piquette-Miller, *Drug Delivery* **2010**, *17*, 365.
[32] V. Gray-Schopfer, C. Wellbrock, R. Marais, *Nature* **2007**, *445*, 851.