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PTD4-apoptin protein and dacarbazine show a synergistic antitumor effect on B16-F1 melanoma *in vitro* and *in vivo*Jia-lu Jin^a, Jing Gong^a, Tie-jun Yin^b, Yan-jun Lu^a, Jing-jing Xia^a, Yu-yuan Xie^a, Yong Di^a, Lei He^a, Jian-li Guo^a, Jun Sun^{a,*}, Mathieu H.M. Noteborn^{a,c}, Shen Qu^a^a Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China^b Comprehensive Department, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China^c Department of Molecular Genetics, Leiden Institute for Chemistry, Leiden University, Leiden, The Netherlands

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ABSTRACT

PTD4-apoptin protein enters cells and harbors tumor-selective cell death activity. Dacarbazine is the mainstay of treatment for malignant melanoma. In this study, we investigated the cytotoxic effect of PTD4-apoptin protein and/or dacarbazine in mouse B16-F1 and human A875 and SK-MEL-5 melanoma cells *in vitro* and by means of a mouse B16-F1 melanoma model *in vivo*. PTD4-apoptin protein inhibits the growth of B16-F1, A875 and SK-MEL-5 melanoma cells in a dose-dependent manner, but not in normal human cell lines WI-38 and L-02. PTD4-apoptin combined with dacarbazine revealed a synergistic cytotoxic effect (coefficient of drug interaction < 1) in all three different tumor cell lines. *In vivo*, PTD4-apoptin protein and dacarbazine alone effectively inhibited the growth of B16-F1 melanoma in C57BL/6 mice. Strikingly, combined PTD4-apoptin/dacarbazine treatment significantly increased the antitumor effect in comparison to the single treatments. As important, a combined PTD4-apoptin/dacarbazine treatment with a 50% reduction of dacarbazine revealed similar antitumor activities, without detectable hematologic side effects. A combined PTD4-apoptin/dacarbazine treatment represents a promising novel efficient and safe anticancer strategy.

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1. Introduction

Worldwide, the incidence and mortality of cutaneous malignant melanoma are continuously increasing (Garbe and Leiter, 2009; MacKie et al., 2009). Localized melanoma has a good prognosis after adequate surgical therapy, but once hematogenous metastasis occurs, the prognosis is very poor despite a large number of clinical trials with various chemotherapeutic agents and biological modifiers (Kasper et al., 2007; Trinh, 2008). The alkylating agent dacarbazine has been approved for treatment of metastatic melanoma and is considered the standard by which other therapies are evaluated (Agarwala, 2009). However, in recent years, randomized trials examining dacarbazine as a single agent have demonstrated its response rates ranging from 6% to 12% without clear impact on overall survival (Agarwala, 2009; Hauschild et al., 2008). To date no alternatives have been reported (Eggermont and Kirkwood, 2004; Lillehammer et al., 2007). Therefore, the development of a new treatment approach is needed.

Apoptin is a small protein derived from chicken anemia virus (CAV), which can selectively induce cell death in tumor cells, but not in normal cells (Noteborn, 2009; Noteborn et al., 1994; Rohn and

Noteborn, 2004; Zhang et al., 2003). In transformed human cells, apoptin becomes selectively phosphorylated. Apoptin forms multimeric complexes, which interact in tumor cells with chromatin structures. Apoptin synthesis in tumor cells results in enhanced tumor suppressor ceramide levels, indicating that sphingolipids play a crucial role in apoptin's tumor-selective cell death. Apoptin induces G2/M cell cycle arrest by interaction with the anaphase promoting complex/cyclosome resulting in p73/PUMA-induced cell death. Apoptin induces cell death independently of the tumor suppressor p53 and can be stimulated by Bcl-2 (Backendorf et al., 2008; Noteborn, 2009; Grimm and Noteborn, 2010). All these features are indicative that apoptin can exert its activity under conditions where most currently used anticancer agents fail (Maddika et al., 2006; Noteborn, 2005).

Based on the above properties, apoptin is expected to be a potential anticancer therapy candidate. It is reported that the expression of apoptin alone or in combination with other (chemo)therapeutics can inhibit tumor cell growth *in vitro* or in mouse bearing tumor models (Lian et al., 2007; Olijslagers et al., 2007; Peng et al., 2007; Van der Eb et al., 2002). To avoid the potential limitations related to exogenous gene transduction (Bagi, 2005; Liu, 2006) and to expand apoptin's antitumor therapy potential, our laboratory developed a novel apoptin protein therapy. The protein transduction domain 4 (PTD4) was fused to the apoptin protein facilitating the apoptin protein to be delivered across cellular membranes in a very

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efficient way (Ho et al., 2001; Sun et al., 2009). PTD4-apoptin significantly inhibited tumor growth *in vivo* without cytotoxic side effects on normal tissue as shown for HepG2, HeLa and SGC7901 tumor xenografted nude mouse models (Sun et al., 2009).

In the present study, we examined whether PTD4-apoptin can enhance the antitumor potential of dacarbazine. Therefore, we investigated the effects of PTD4-apoptin protein alone or in combination with dacarbazine *in vitro* and examined their anticancer efficacy and hematologic side effects *in vivo*. PTD4-apoptin and dacarbazine reveal a synergistic antitumor effect with reduced side effects.

2. Material and methods

2.1. Anticancer drugs

Dacarbazine was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and was activated by exposure to light for 1 h before it was used *in vitro*. Next, dacarbazine was dissolved in 1 M hydrochloric acid according to the supplier's instructions and diluted to various concentrations with culture medium. When dacarbazine was interperitoneally injected in tumor-bearing or tumor-free mice, it was dissolved in 0.9% NaCl solution to a specific concentration. PTD4-apoptin protein and PTD4-EGFP protein were prepared according to the methods described by Sun et al. (2009). Before the start of the *in-vitro* experiments, the lyophilized powders of PTD4-apoptin proteins and PTD4-EGFP proteins were dissolved in sterile phosphate-buffered saline (PBS). For *in-vivo* experiments, PTD4-apoptin protein and PTD4-EGFP protein was dissolved in PBS + 30% glycerol solution and applied carefully on the tumor-bearing epidermis.

2.2. Cell culture

The mouse melanoma cell line B16-F1, the human melanoma cell line A875, normal human embryonic lung fibroblast WI-38 cell line, and human normal hepatocyte L-02 cell line were purchased from the China Center for Type Culture Collection (Wuhan, PR China). The human melanoma cell line SK-MEL-5 was kindly provided by professor Xiongwen Wu, Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, PR China. B16-F1, A875 and SK-MEL-5 cells were all cultured in DMEM medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. WI-38 and L-02 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ at 37 °C. WI-38 cells were used between passages 6 and 12.

2.3. Cell viability assay and combined effect evaluation

B16-F1 cells, A875 cells or SK-MEL-5 cells or L-02 cells were plated in 96-well plates at a density of 3×10^3 cells per well, respectively. WI-38 cells were plated in 96-well plates at a density of 2.5×10^3 cells per well. After 24 h, PTD4-apoptin protein and/or dacarbazine was added into wells with an indicated concentration. PTD4-EGFP, as the negative control of PTD4-apoptin protein, was also added into wells in the same way. After incubation for 48 h, 20 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (5 mg/ml in PBS, Sigma, St. Louis, MO, USA) was added into each well. After incubation at 37 °C for 4 h, the medium was discarded completely, and 150 µl dimethyl sulfoxide was added into each well to dissolve the formazan crystals of the cell. Next, its absorbance was measured by a microplate reader (Tecan Sunrise, Mannedorf, Switzerland) at 570 nm as detection wavelength and 630 nm as reference wavelength. Each experiment was performed with five parallel observations and repeated three times in an independent way.

The survival ratio (%) was calculated using the following equation: survival ratio (%) = (average absorbance of treated group/average absorbance of control group) × 100. IC50 was defined as the drug concentration at which 50% of the cell growth was inhibited and was analyzed by means of SigmaPlot 8.0.

The coefficient of drug interaction (CDI) was used to analyze the nature of the drug interaction between PTD4-apoptin protein and dacarbazine (Cao and Zhen, 1989; Xu et al., 2007). CDI was calculated as follows: $CDI = AB / (A \times B)$. According to the absorbance of each group, AB is the ratio of the 2-drug combination group to the control group in OD570/630, and A or B is the ratio of the single drug group to the control group in OD570/630. $CDI < 1$ indicates synergism, $CDI < 0.7$ indicates a significantly synergistic effect, $CDI = 1$ indicates additivity and $CDI > 1$ indicates antagonism.

2.4. DAPI staining

B16-F1 cells were seeded in 6-well plates containing cover slips at a concentration of 1.5×10^5 cells per well. After culturing for 24 h, 15 µg/ml PTD4-apoptin or 250 µM dacarbazine alone or a 2-drug combination was added into the wells. The cells were incubated during 48 h. Next, the morphology of cells was monitored under an inverted light microscope. Then the cover slips were washed twice with PBS, fixed with 4% paraformaldehyde for 20 min, washed with PBS and finally incubated with 1 µg/ml DAPI (4',6'-diamidino-2-phenylindole, Roche Applied Science, Indianapolis, IN, USA) for 15 min. After washing with PBS, cells were analyzed by means of fluorescent microscopy (Olympus, Tokyo, Japan) with a peak excitation wave length of 340 nm. The characteristics of nuclear morphological changes of cell death such as the condensation of nuclear chromatin and fragmentation were examined.

2.5. Annexin-V/propidium-iodide (PI) double staining assay

B16-F1 cells were seeded in 6-well plates at a concentration of 1.5×10^5 cells per well and incubated with 15 µg/ml PTD4-apoptin or 250 µM dacarbazine alone or a 2-drug combination. After 48 h, cells were collected, washed with PBS for 2 times and suspended with binding buffer containing 2 µl FITC-labeled Annexin-V and 5 µl PI according to the instructions of the supplier's kit (Kaigi, Nanjing, China). After at least 15 min of incubation, the cells were examined by flow cytometric analysis (BD LSR II Flow Cytometer; BD Biosciences, San Jose, CA, USA). Cells in early stages of apoptosis were Annexin-V positive; whereas, cells that were both Annexin-V and PI positive were regarded to be in a late stage of apoptosis.

2.6. In-vivo melanoma tumor model and treatments

Female C57BL/6 mice (7–8 weeks old) were obtained from the Experimental Animal Center of the Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. All animal studies were carried out in accordance with the "Guide for the Care and Use of Laboratory Animals" and approved by the Institute of Laboratory Animal of Hubei Province. To establish the tumor model, 2×10^6 B16-F1 cells were suspended in 100 µl PBS buffer and injected subcutaneously into the right hind groin of each mouse.

Firstly, the inhibitory effect of PTD4-apoptin on the B16-F1 melanoma growth in comparison to PTD4-EGFP protein and PBS was examined. We established 12 tumor-bearing mice, and after the tumors were clearly visible, the mice were divided randomly into three groups. During a treatment period of 9 days, every day the tumor-bearing epidermis was applied with 100 µg PTD4-apoptin, PTD4-EGFP or PBS.

Subsequently, we detected the inhibitory effect of the combination of PTD4-apoptin and dacarbazine. The tumors were established as described above. After the tumors were clearly visible, 35 tumor-

bearing mice were divided randomly into five groups (7 mice in each group): ① PTD4-apoptin protein treatment group (100 µg/tumor/day, every day) ② dacarbazine treatment group (70 mg/kg, once every 2 days, five times) ③ “full dose” drug combination group (dacarbazine, 70 mg/kg, once every 2 days, five times + PTD4-apoptin protein, 100 µg/tumor/day, every day) ④ “half dose” drug combination group (dacarbazine, 35 mg/kg, once every 2 days, five times + PTD4-apoptin protein, 100 µg/tumor/day, every day) and ⑤ control group (PBS, on the tumor-bearing epidermis, every day). PTD4-apoptin or PBS preparations were carefully applied on the tumor-bearing epidermis, whereas dacarbazine was intraperitoneally injected in tumor-bearing mice. The control, single and combined treatments lasted for 15 days.

2.7. Analysis of an *in-vivo* antitumor effect

The antitumor effect of PTD4-apoptin protein and/or dacarbazine treatment was determined as follows. The length and width of the tumors were measured using calipers before the experiment and measured every 2 days till the end of the experiments. The tumor volume was calculated according to the following formula: volume = length × width² × 0.52. When the experiment was terminated, the mice were killed by cervical dislocation.

The treated mice, within the experiment examining the inhibitory effect of PTD4-apoptin protein in comparison to PTD4-EGFP and PBS, were sacrificed after 9 days. The tumor tissue from each mouse was excised, photographed and weighed.

After a 15-day treatment, the mice within the *in-vivo* combinational experiments were all sacrificed. Two mice from each group were randomly chosen and a part of their tumor tissue covered by epidermal tissue was excised and fixed for immunohistochemistry to detect the possible presence of PTD4-apoptin protein. The tumors from the other 5 mice were excised, photographed, weighed and fixed for Tdt-mediated dUTP nick-end labeling (TUNEL) assays. The growth inhibition rate was calculated according to the formula: the inhibition rate = (mean tumor weight of control group – mean tumor weight of treatment group)/(mean tumor weight of control group) × 100%.

2.8. Immunohistochemistry and TUNEL staining

The excised tumors or tumors covered with epidermal tissue were fixed with 4% paraformaldehyde, and paraffin-embedded sections were prepared for carrying out hematoxylin and eosin (HE) staining, TUNEL assays or immunohistochemistry. HE staining was performed by routine methods. TUNEL staining was performed using the *in-situ* apoptosis detection kit POD, according to the supplier's protocol (Roche Applied Science, Mannheim, Germany). Immunohistochemical staining for His-tagged PTD4-apoptin by anti-His antibodies (GE Healthcare, Piscataway, NJ, USA) was performed according to the routine method to show the presence of PTD4-apoptin protein. TUNEL staining and immunohistochemistry were both colored by means of AEC (3-amino-9-ethylcarbazole).

2.9. Assay for evaluating hematologic side effects

The experiments for determining possible side effects by PTD4-apoptin and/or dacarbazine were carried out in tumor-free animals. 25 female C57BL/6 mice were divided randomly into five groups (5 mice in each group). The treatment regimen was the same as described for the tumor-bearing mice models. Fifteen days after treatment, peripheral blood cells were collected from mice by cardiac puncture into K₂EDTA-containing tubes and analyzed by Automatic Blood Chemistry Analyzer (sysmex, xs-800i, Japan). The following blood parameters were measured and compared with normal values: red and white blood cell and platelet count and determination of the hemoglobin level. A decrease in hemoglobin, platelets and white

blood cells in comparison to normal value indicates bone marrow suppression.

2.10. Statistics

The statistical significance of differences was evaluated by the *t*-test using SPSS software (version 13.0). A *P* value of less than 0.05 (*) or less than 0.01 (**) was considered to be significant. Data presented in the figures represent the mean ± standard error.

3. Results

3.1. Anti-proliferation effect of PTD4-apoptin protein and/or dacarbazine on mouse and human melanoma cells *in vitro*

Under *in-vitro* conditions, we have examined the cytotoxic effects of PTD4-apoptin and dacarbazine alone or combined with each other on B16-F1, A875, and SK-MEL-5 tumor cells. The effects of PTD4-apoptin or dacarbazine on normal human WI-38 and L-02 cells and the cytotoxicity of PTD4-EGFP protein on melanoma B16-F1 and A875 cells or normal WI-38 and L-02 cells were examined. Firstly, the inhibitory effect of increasing concentrations of PTD4-apoptin, PTD4-EGFP protein (5, 10, 15, 20, and 25 µg/ml) or dacarbazine (62.5, 125, 250, 500, 1000, and 2000 µM) on the cell viability of B16-F1, A875, SK-MEL-5, WI-38 and L-02 cells was determined by means of MTT assay. The results indicated that both PTD4-apoptin and dacarbazine inhibit cell growth in a dose-dependent manner (Fig. 1A, C). The IC₅₀ of PTD4-apoptin protein in B16-F1 cells, A875 cells and SK-MEL-5 cells was 19.3 ± 4.3 µg/ml, 17.5 ± 5.1 µg/ml and 14.9 ± 4.4 µg/ml, respectively. The IC₅₀ of dacarbazine in the same cells was 259.5 ± 22.7 µM, 287 ± 20.9 µM and 380 ± 29.8 µM, respectively. Although less sensitive than tumor cells, normal human WI-38 and L-02 cells also showed signs of growth inhibition upon dacarbazine treatment (1 C), but normal cells treated with PTD4-apoptin protein did not show any significant signs of growth inhibition (Fig. 1A). The IC₅₀ of dacarbazine in normal cells WI-38 and L-02 was 526.46 ± 37.53 µM and 366.94 ± 28.72 µM, respectively. B16-F1 and A875 tumor cells and WI-38 and L-02 normal cells treated with PTD4-EGFP protein did not show any significant signs of growth inhibition (Fig. 1B). The results indicate that PTD4-apoptin protein can selectively act on tumor cells, whereas chemotherapy agent dacarbazine has a rather limited selectivity.

Three doses of PTD4-apoptin protein (5, 10 and 15 µg/ml) were used in combination with different concentrations of dacarbazine (62.5, 125, 250, 500, and 1000 µM) mixed at a fixed ratio (1:1, v/v), respectively. The obtained MTT-results showed that PTD4-apoptin protein significantly increased the cytotoxicity of dacarbazine in mouse melanoma B16-F1 cells (Fig. 2A). For example, in the presence of 5, 10 and 15 µg/ml PTD4-apoptin, the IC₅₀ of dacarbazine in B16-F1 cells reduced from 259.5 ± 22.7 µM to 127.1 ± 18.5 µM, 86.4 ± 13.9 µM and 43.5 ± 8.8 µM, respectively (Fig. 2A).

To analyze whether the synergistic inhibitory effect of combined PTD4-apoptin protein and dacarbazine also holds true for human melanoma cells, the human melanoma cell lines A875 and SK-MEL-5 were treated with PTD4-apoptin and dacarbazine. The obtained MTT-results indicate that also in the two human tumor cell lines PTD4-apoptin protein significantly increased the cytotoxicity of dacarbazine (Fig. 2B, C). In the presence of 5, 10 and 15 µg/ml PTD4-apoptin protein, the IC₅₀ of dacarbazine in A875 cells was reduced from 287 ± 20.9 µM to 124 ± 17.3 µM, 83.6 ± 10.7 µM and 40.3 ± 7.9 µM, respectively. The IC₅₀ of dacarbazine in SK-MEL-5 cells was reduced from 380 ± 29.8 µM to 241 ± 12.3 µM, 101.6 ± 8.6 µM and 34.3 ± 5.7 µM.

Finally, the coefficient of drug interaction (CDI) was determined to evaluate the nature of the drug interaction. The results showed that in all analyzed cases the CDI was <1, which means a synergistic

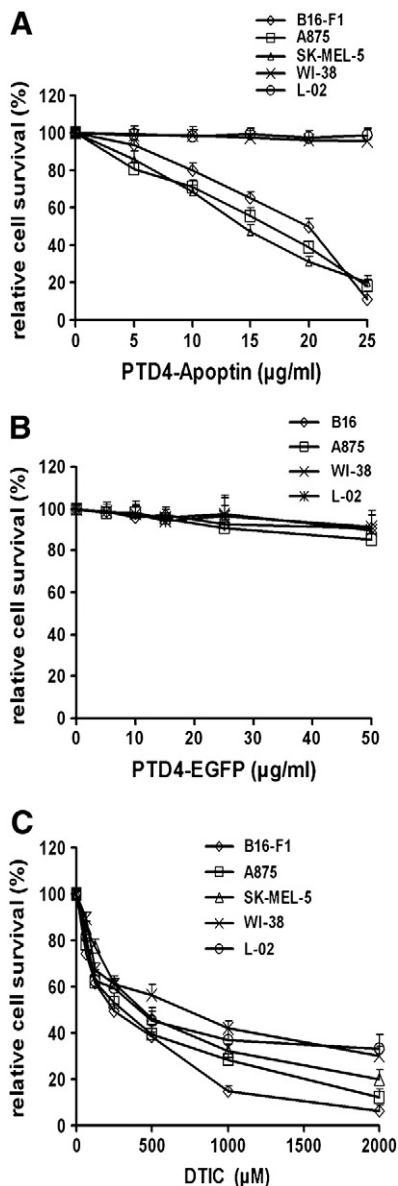


Fig. 1. The dose–effect curve of PTD4-apoptin protein, PTD4-EGFP protein or dacarbazine (DTIC) on the cell viability in melanoma cells B16-F1, A875 and SK-MEL-5 or normal human cells WI-38 and L-02. The cell lines were incubated with (A) various concentrations of PTD4-apoptin protein, with (B) various concentrations of PTD4-EGFP protein, with (C) various concentrations of DTIC during 48 h, respectively. The cell viability was determined using MTT assay. Data are presented as mean \pm S.D. (error bar) of three independent experiments.

inhibitory effect between PTD4-apoptin protein and dacarbazine on the three cell lines. Under some conditions for B16-F1 cells (Fig. 2A'), the CDI was even <0.7 . For example, for the combination of 10 $\mu\text{g/ml}$ PTD4-apoptin and 500 μM dacarbazine, or the combination of 15 $\mu\text{g/ml}$ PTD4-apoptin and 250 μM , 500 μM , or 1000 μM dacarbazine, the CDI was always <0.7 , which showed a synergistic inhibitory effect between PTD4-apoptin protein and dacarbazine. A CDI <0.7 was also found for certain ratios of PTD4-apoptin and dacarbazine in A875 cells and SK-MEL-5 cells (Fig. 2B', C').

3.2. Effects of PTD4-apoptin protein and/or dacarbazine on B16-F1 cell morphology

Next, we examined the morphological effects in B16-F1 cells triggered by the various treatment strategies. Firstly, the cells were incubated for 48 h with 15 $\mu\text{g/ml}$ PTD4-apoptin protein, 250 μM

dacarbazine, a combination, or untreated. As shown in Fig. 3A (a–d), the untreated B16-F1 cells grew well and the cytoskeletons were clearly detectable. Cells treated with dacarbazine were equivocal, and a few cells were swollen or broken. After treatment with PTD4-apoptin, some cells were swollen and even broken. A large proportion of cells treated with the 2-drug combination underwent either swelling or were broken.

In addition, we examined the changes in the nuclear morphology by staining with DAPI. As shown in Fig. 3A (e–h), untreated B16-F1 cells demonstrated homogeneously distributed chromatin within their nuclei, while treated cells presented the morphological features of apoptotic cells in different degrees, such as chromatin condensation and nuclear fragmentation. Most of the cells treated with the dacarbazine/PTD4-apoptin revealed abundant cell death features. Cells treated with PTD4-apoptin show a mediate amount of dead cells, whereas relatively few killed cells were observed in cells treated with dacarbazine. We conclude that a combined PTD4-apoptin/dacarbazine treatment is more potent in inducing cell death in comparison to the single treatments.

3.3. Increased apoptosis rate induced by a combination of PTD4-apoptin protein and dacarbazine in B16-F1 cells

To further specify the increased cell death rate induced by PTD4-apoptin/dacarbazine, treated cells were analyzed by means of an Annexin-V/PI staining assay. Compared with the mock-treated control group, the proportion of apoptotic cells increased upon 48 h exposure to 250 μM dacarbazine, 15 $\mu\text{g/ml}$ PTD4-apoptin protein or a combination with the total apoptosis rate of $13\% \pm 1.7\%$, $21.8\% \pm 1.6\%$, and $59.5\% \pm 5\%$, respectively (Fig. 3C). Fig. 3B shows that the proportion of both early and late apoptotic cells is the highest for the combination treatment, whereas dacarbazine revealed the slightest early/late apoptosis induction.

The morphological analysis and the Annexin-V/PI staining assay indicated that PTD4-apoptin protein combined with dacarbazine achieved the most prominent apoptotic-inducing effect in B16-F1 melanoma-derived tumor cells.

3.4. In vivo, PTD4-apoptin/dacarbazine combinatorial treatment results in enhanced anticancer activities

To examine the anticancer effects of a combination strategy based on PTD4-apoptin and dacarbazine under *in-vivo* conditions, 12 C57BL/6 mice bearing subcutaneous melanoma tumors were randomly divided into 3 groups and treated with PBS, PTD4-EGFP or PTD4-apoptin protein as described in the Materials and methods section. After 9 days of treatment, the tumors were excised and weighed. The results showed that the tumor weights of PTD4-apoptin-treated mice were about one third of the tumor weights of control mice treated with PBS or PTD4-EGFP ($P < 0.01$). The tumor weights of the PBS and PTD4-EGFP control groups had no significant difference ($P = 0.852$) in comparison to each other (Fig. 4A). The excised tumors of each treated group are shown in Fig. 4B.

The results indicate that PTD4-apoptin inhibits the growth of the subcutaneous melanomas effectively whereas PTD4-EGFP did not. Therefore, one can conclude that the observed tumor growth inhibition is specifically due to PTD4-apoptin protein and not just due to an immunogenic effect against the transduced recombinant protein.

Next, we examined whether the synergistic cytotoxic effect of PTD4-apoptin and dacarbazine observed *in vitro* could be reproduced under *in-vivo* conditions. To that end, 35 female C57BL/6 bearing subcutaneous melanoma tumors were randomly divided into 5 groups and treated with PTD4-apoptin protein and/or dacarbazine. During 15 days of treatment, the size of the tumors was analyzed every 2 days. As shown in Fig. 4C, the tumor growth was significantly inhibited in all treatment groups ($P < 0.05$). The tumor growth of

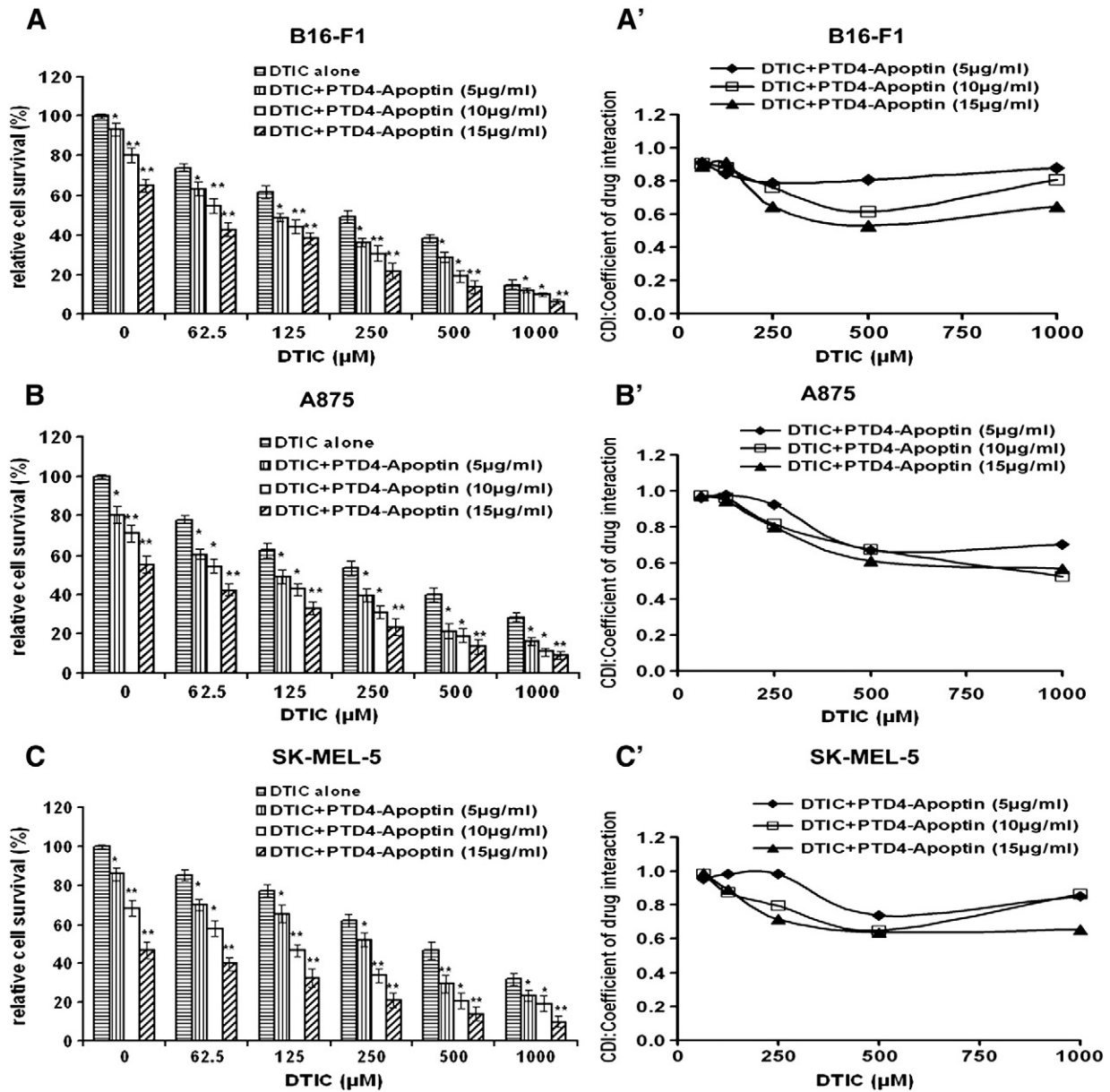


Fig. 2. The inhibitory effects of PTD4-apoptin protein combined with dacarbazine (DTIC) on cell viability of mouse and human melanoma cells and the coefficient of drug interaction (CDI). Panel A shows the data for B16-F1 cells, B for A875 cells and C for SK-MEL-5 cells. The various cells were incubated with 5, 10, and 15 μg/ml PTD4-apoptin protein combined with 62.5, 125, 500 or 1000 μM DTIC during 48 h. The cell viability was determined using MTT assay. Data are presented as mean ± S.D. (error bar) of three independent experiments. **P<0.01 and *P<0.05 compared with DTIC treatment. (A', B', C') The CDI of PTD4-apoptin protein combined with DTIC on (A') B16-F1 cells, (B') A875 cells and (C') SK-MEL-5 cells. CDI<1 means synergistic inhibitory effect between PTD4-apoptin protein and dacarbazine, whereas CDI<0.7 indicates a significantly synergistic inhibitory effect.

PTD4-apoptin protein treatment group was significantly lower than that of the dacarbazine group ($P<0.05$). The tumor growth of the two 2-drug combination groups was significantly lower than that in each single treatment groups ($P<0.05$). The growth of the tumors treated with PTD4-apoptin and a 50% reduced dacarbazine was not significantly different in comparison to tumors treated with PTD4-apoptin and high dose dacarbazine ($P=0.237$).

Fifteen days after treatment, the mice were killed and the tumors were excised. Macroscopic analysis also showed that the size of tumors treated with PTD4-apoptin and reduced dacarbazine or high dose dacarbazine was significantly smaller than those treated with a single drug or of the control group (Fig. 4D). In addition, we determined the tumor growth inhibition rate by weighing the excised tumor according to the formula described in the Materials and methods section. The tumor growth inhibition rate in the dacarbazine and PTD4-apoptin treatment groups was 59.1% and 70.4%, respective-

ly. The tumor growth inhibition rate of the PTD4-apoptin and “50% or 100%” dacarbazine treatment group was 90.2% and 93.2%, respectively.

3.5. PTD4-apoptin protein penetrates into tumor cells via epidermal tissue

To ascertain the contribution of PTD4-apoptin protein to the observed tumor growth reduction, we determined whether the PTD4-apoptin protein could be transduced into the melanoma tumor tissue via epidermal tissue. To that end, we analyzed the presence of PTD4-apoptin protein in sections derived from each treatment group consisting of the epidermis covering tumor tissue by immunohistochemistry. HE staining showed the characteristic morphology of both the epidermal and tumor tissue (Fig. 5A). Antibodies directed against the histidine tag of PTD4-apoptin, indicated its presence in the epidermal and tumor tissue. PTD4-apoptin protein was transduced

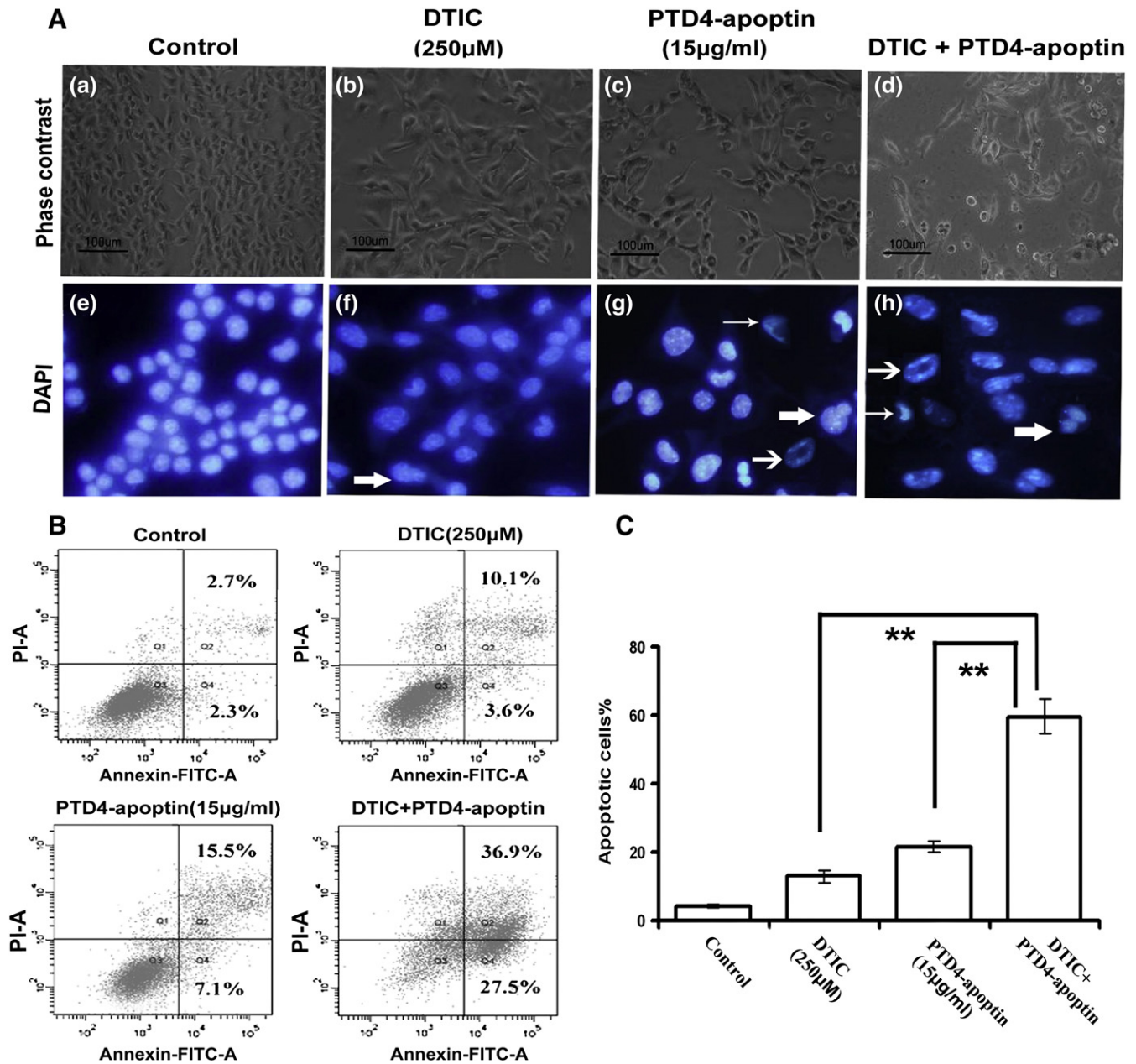


Fig. 3. Morphological changes and apoptosis in B16-F1 cells treated with 250 μM dacarbazine (DTIC), 15 μg/ml PTD4-apoptin protein, a 2-drug combination or untreated (control) for 48 h. (A) Phase-contrast micrographs (a, b, c, d, magnification: $\times 200$) and fluorescence micrographs (e, f, g, h) showed the morphological changes in B16-F1 cells: (a, e) control; (b, f) 250 μM DTIC; (c, g) 15 μg/ml PTD4-apoptin protein; and (d, h) a 2-drug combination. B16-F1 cells subjected to DAPI staining, which is illustrative for chromatin condensation, nuclear fragmentation or apoptotic body formation (indicated like three different types of arrow), all indicative for apoptosis. (B) Apoptosis rate determined by Annexin-V/PI staining. Early apoptotic cells are Annexin-V positive, PI-negative (lower right), whereas late apoptotic cells are Annexin-V- and PI-positive (upper right). (C) The apoptotic rates of B16-F1 cells treated with PTD4-apoptin protein and/or DTIC for 48 h. The apoptotic rate of the 2-drug combination was significantly higher than that of DTIC alone or PTD4-apoptin alone (** $P < 0.01$).

into the tumors via the epidermal tissue of the mice treated with PTD4-apoptin protein alone or in combination with dacarbazine. Tumor tissues derived from the negative control group and dacarbazine-only group were, as expected, negative (Fig. 5B).

3.6. *In vivo*, PTD4-apoptin/dacarbazine treatments are cytotoxic via induction of apoptosis

To investigate the underlying process of the antitumor effect in tumor-bearing C57BL/6 mice treated with PTD4-apoptin protein, dacarbazine or the combination, sections of all tumors were prepared for detection of the level of cell death by means of TUNEL assay. The results showed that tumors treated with both PTD4-apoptin/dacarbazine

regimens contained many TUNEL-positive cells. Tumors treated with PTD4-apoptin protein also contained a relatively moderate level of dead cells, whereas dacarbazine-treated tumors contained a few apoptotic cells. Control tumors did not reveal almost no TUNEL-positive cells (Fig. 5C).

Therefore, we conclude that *in vivo* PTD4-apoptin protein combined with dacarbazine induces an enhanced inhibition of melanoma tumor growth via induction of apoptosis.

3.7. Hematologic side effects

Finally, we examined whether the various PTD4-apoptin and/or dacarbazine treatment regimens cause hematologic side effects in the

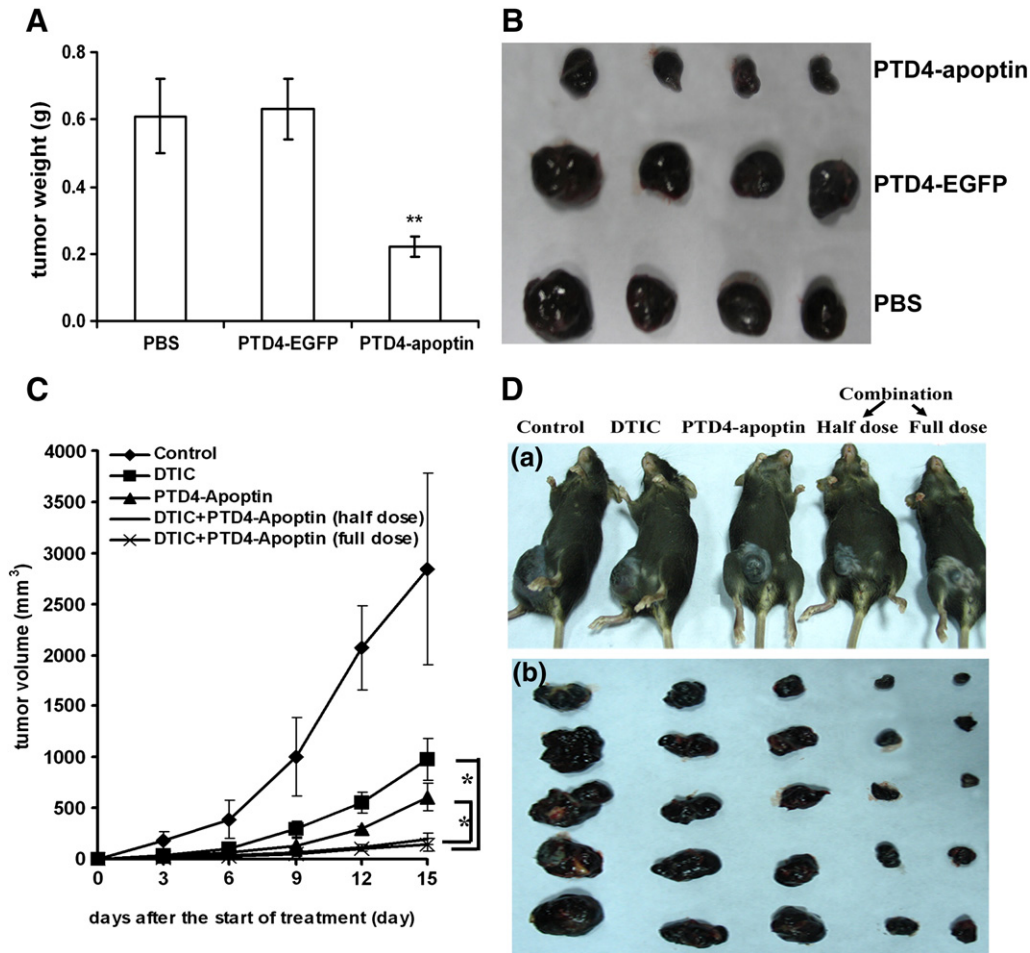


Fig. 4. In B16-melanoma bearing mice PTD4-apoptin protein enhances antitumor activity of dacarbazine (DTIC). (A, B) Inhibition of B16-F1 melanoma tumor volume by PTD4-apoptin protein in comparison to PTD4-EGFP protein or PBS treatment. The tumor mice were treated every day with PTD4-apoptin protein, PTD4-EGFP and PBS during 9 days. (A) Diagrams showed the mean tumor volumes \pm standard deviation of the excised tumors after the treatment regimens with PTD4-apoptin, PTD4-EGFP or PBS. (B) Photographs of the excised tumors of all the treated groups and of the control group. (C) Volumes of the melanoma tumor tissue during treatment with DTIC, PTD4-apoptin protein, “half dose” drug combination, “full dose” drug combination or control. The tumor size was measured every 2 days. The results are expressed as the mean \pm S.D. from 7 tumor-bearing mice in each group. * $P < 0.05$ compared between a single treatment and combined treatment. (D) (a) Macroscopic appearances in drug-treated mice or control ones. (b) Excised tumors of all the treated groups and of the control group. Note the significant decrease in tumor volume associated with “half dose” and “full dose” drug combination treatment in comparison to the control and mono-treated ones.

treated mice. Tumor-free mice were treated with dacarbazine and/or PTD4-apoptin as described for the tumor-bearing mice experiments. Fifteen days after treatment, blood was collected by heart puncture. As shown in Table 1, upon dacarbazine alone or a combination treatment with high dose dacarbazine and PTD4-apoptin a decrease in hemoglobin, platelets and white blood cells was observed in comparison to the control-treated group. PTD4-apoptin protein alone and the combination treatment based on 50%-reduced dacarbazine and PTD4-apoptin had no significant negative effect on the hemoglobin level, as well as on the amount of platelets and white blood cells. The obtained results suggest that high concentrations of dacarbazine resulted in bone marrow suppression, whereas PTD4-apoptin alone and “half dose” combinatorial treatment do not.

4. Discussion

Here, we explored new approaches for treating melanoma. Our data show for the first time that a combinatorial treatment of B16-F1 melanoma *in vitro* and *in vivo* based on PTD4-apoptin protein and dacarbazine represents an effective antitumor strategy without detectable side effects. Dacarbazine used as single-agent therapy is the approved therapy for patients with metastatic melanoma, but its response rates are disappointing and the effect is not sustained

(Lillehammer et al., 2007). Other single-agent chemotherapies such as temozolomide, taxanes, cisplatin, carboplatin or immunotherapeutic agents such as interferons and interleukin-2 achieve similar results as dacarbazine (Hauschild et al., 2008; Yang and Chapman, 2009). Combination chemotherapy regimens such as dartmouth regimen (dacarbazine/carmustine/cisplatin/tamoxifen), CVD (cisplatin/vinblastine/dacarbazine) or biochemotherapy regimens containing interferon- α , interleukin-2 and 3 different cytotoxic drugs (cisplatin, vinblastine, and dacarbazine) have no overall survival benefit or are highly toxic compared to dacarbazine (Trinh, 2008; Yang and Chapman, 2009).

In vitro, PTD4-apoptin protein alone had a dose-dependent cytotoxicity on the growth of mouse B16-F1 melanoma cells as well as on human A875 and SK-MEL-5 melanoma cells. However, PTD4-apoptin had no detectable negative effect on the analyzed normal cells. The obtained CDI results showed that PTD4-apoptin protein acts synergistically with dacarbazine in the analyzed mouse and two human melanoma cell lines. These results suggest that a combinatorial treatment consisting of PTD4-apoptin local treatment and systemic delivery of dacarbazine can be more beneficial than the separated single treatments.

Indeed, our *in-vivo* data confirmed our *in-vitro* observed synergistic behavior between PTD4-apoptin protein and dacarbazine

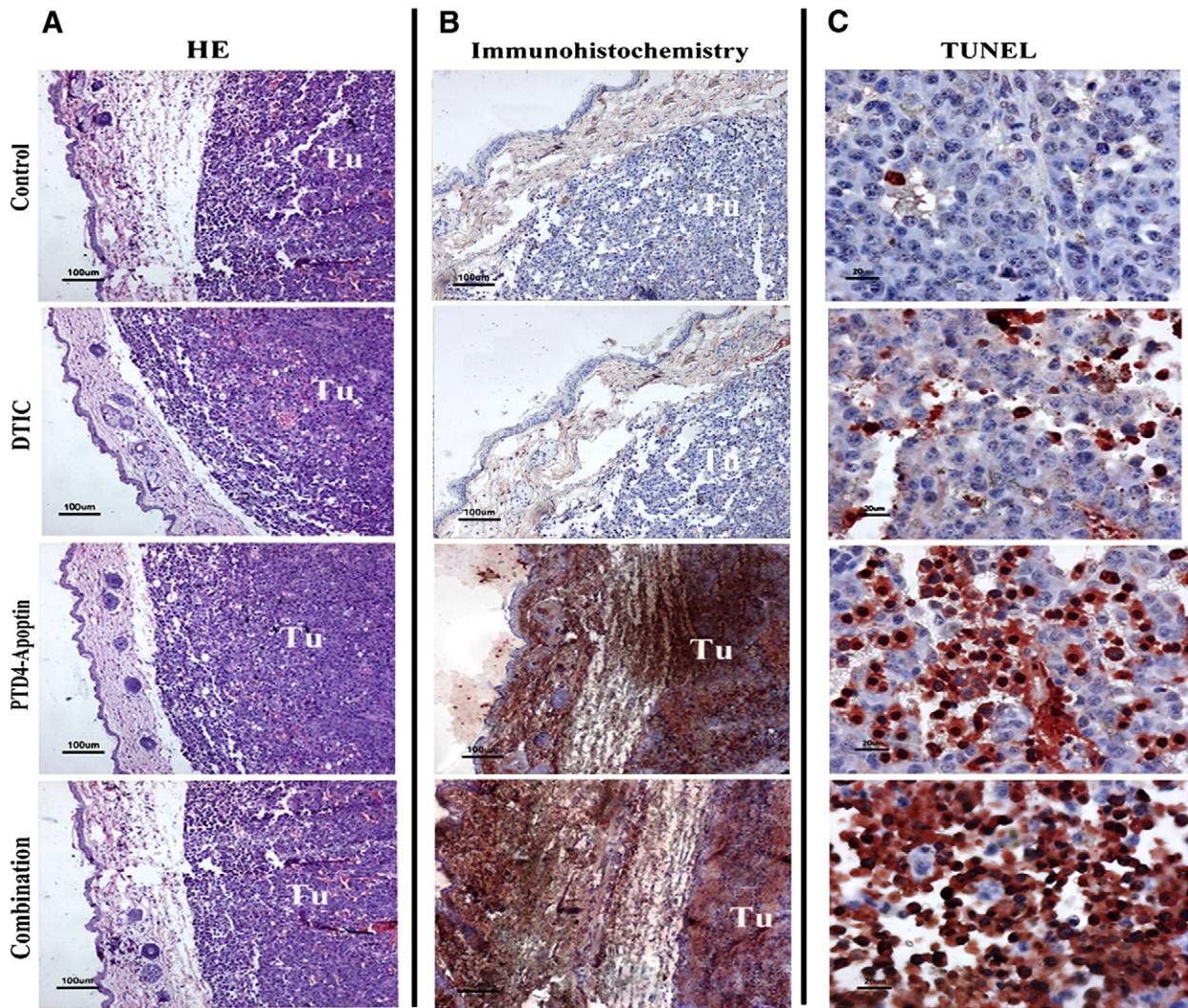


Fig. 5. PTD4-apoptin protein penetrates through normal and tumor tissue resulting in apoptosis in tumor tissue only. (A) HE staining showed the characteristic morphology of both the epidermal and tumor tissue (Tu). (Magnification: $\times 100$). (B) Immunohistochemistry analysis shows the presence of PTD4-apoptin protein in both epidermal and melanoma tissues when supplied on the epidermis only or in combination with intraperitoneally injected dacarbazine (DTIC). (Magnification: $\times 100$). (C) TUNEL analysis reveals that PTD4-apoptin enhances the apoptotic activity of DTIC. The cells undergoing apoptosis are colored red. (Magnification: $\times 400$).

treatment. Combined PTD4-apoptin/dacarbazine treatments suppressed the growth of the subcutaneous melanomas significantly higher than either mono-therapy. Lowering the dose of dacarbazine to 50% did not result in a significant reduction of antitumor activity of the combinatorial therapy. One can conclude that also under *in-vivo* conditions apoptin's tumor-selective cell death characteristics are enhancing the dacarbazine cell killing activity.

In vivo, TUNEL and Annexin-V/PI staining showed that the percentage apoptotic cells were significantly higher in tumor tissue treated with the both PTD4-apoptin and dacarbazine versus the single

drug treatment groups. All these results indicate that PTD4-apoptin/dacarbazine combination treatments inhibit tumor growth more effectively than single treatments by increasing the apoptosis induction levels.

In a previous study, Sun et al. (2009) have demonstrated that local treatment with PTD4-apoptin results in the presence of PTD4-apoptin in e.g. liver, brain, kidney and lung tissue of nude mice without resulting in any detectable cytotoxic effect. In this study, we examined possible hematologic side effects of treatments based on PTD4-apoptin and/or dacarbazine of tumor-free mice. Dacarbazine harbors a

Table 1
Blood cell and hemoglobin parameters of tumor-free mice after administration of dacarbazine (DTIC), PTD4-apoptin protein or its combinations. The experimental details are described in the Materials and methods section.

Parameter	Measured value					Normal value
	Control	DTIC	PTD4-apoptin	DTIC + PTD4-apoptin		
				Full dose	Half dose	
Leucocytes ($\times 10^9/l$)	5.6 ± 0.4	3.3 ± 0.7	6.0 ± 0.6	4.4 ± 0.6	5.6 ± 0.3	5.1–11.6
Erythrocytes ($\times 10^{12}/l$)	9.1 ± 0.3	6.0 ± 1.3	9.8 ± 0.3	7.3 ± 0.4	9.2 ± 0.5	8.7–10.5
Hemoglobin (g/l)	134.7 ± 3.7	95.5 ± 6	142 ± 5	108.3 ± 7.1	129 ± 6.2	120–150
Platelets ($\times 10^9/l$)	921.7 ± 50	600 ± 83	939 ± 23	865.8 ± 62	908.3 ± 69	100–1000

main side effect consisting of bone marrow suppression (Wolf et al., 2006). PTD4-apoptin alone or a combination treatment of PTD4-apoptin and 50% reduced dacarbazine had no detectable effect on bone marrow suppression. A high dose of dacarbazine treatment alone or combined with PTD4-apoptin revealed severe bone-marrow suppression.

In conclusion, PTD4-apoptin protein combined with dacarbazine showed synergistic inhibitory effects on the growth of mouse melanoma *in vitro* and *in vivo*. As important, *in-vivo* PTD4-apoptin combined with “half dose” dacarbazine regimen led to synergistic antitumor effects without side effects on bone marrow suppression. Our preclinical observations indicate that a combinatorial therapy based on PTD4-apoptin protein and dacarbazine is a promising efficient and safe anticancer strategy. The PTD4-apoptin protein approach can be used for topical applications, which implies that our method is limited to treatments of cancers such as squamous cell carcinoma or precancerous skin lesions.

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