Effects of VBMDMP on the reversal of cisplatin resistance in human lung cancer A549/DDP cells

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Received July 24, 2014; Accepted October 20, 2014

DOI: 10.3892/or.2014.3607

Abstract. Tumor drug resistance is a major obstacle to cancer chemotherapy. We previously constructed a fusion protein based on two tumstatin-derived sequences named recombinant VBMDM (rVBMDMP). We preliminarily confirmed its inhibition of HUVEC and colon cancer cell growth. The present study further systematically observed the inhibitory effect of rVBMDMP on lung cancer cell growth and analyzed a possible mechanism to provide a theoretical basis for the development of new antitumor protein drugs. The effect of rVBMDMP on human lung adenocarcinoma (A549) and cisplatin-resistant human lung adenocarcinoma (A549/DDP) cell proliferation was evaluated by MTS assay. Hoechst 33342 staining performed together with fluorescence microscopy and immunoblot analysis were used to examine the effects of rVBMDMP on the apoptosis of A549/DDP cells. A protein phosphorylation chip was used to identify changes in rVBMDMP-induced signaling protein phosphorylation. Changes in the phosphatidylinositol 3 kinase (PI3K)/Akt signal transduction pathway and expression of multidrug resistance protein (MRP-2)-related molecules following rVBMDMP treatment in A549/DDP cells were evaluated by western blot analysis. A lung cancer xenograft model was used to evaluate the reversal effect of rVBMDMP on drug-resistance of A549/DDP cell tumors to cisplatin *in vivo*. The results demonstrated that rVBMDMP increased the phosphorylation of 79 signaling proteins, including focal adhesion kinase (FAK), caspase-6, Fas, FasL and FAF1 and downregulated 30 signaling proteins, including integrin αV, integrin β3, PI3K/Akt, NF-κB and MRP-2 compared with the controls. rVBMDMP also increased the sensitivity of A549 and A549/DDP cells to cisplatin and directly induced apoptosis, which may be related to MRP-2 and Bcl-2 downregulation. The effects of growth inhibition and apoptosis induction of rVBMDMP on A549/DDP cells may be related to the inhibition of integrin αVβ3 and PI3K/Akt protein phosphorylation. Finally, we observed an increase in cancer cell sensitivity to cisplatin by rVBMDMP using the A549/DDP cell xenograft model in nude mice. Our study suggests that rVBMDMP may be an effective potential chemotherapy sensitizer and may be a viable drug candidate in anticancer therapies.

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Key words: lung carcinoma, recombinant vascular basement membrane-derived multifunctional peptide, polypeptide drug, reversal of multidrug resistance

Introduction

Based on the strategy of targeting both proliferating tumor cells and endothelial cells in our previous studies, we constructed a fusion protein of the human IgG3 upper hinge region and 2 tumstatin-derived specific sequences, which we named vascular basement membrane-derived multifunctional peptide (VBMDMP) (1). Recombinant VBMDMP (rVBMDMP) was found to exhibit anti-proliferation and anti-angiogenic activities and to significantly inhibit tumor growth and metastasis in a mouse lung carcinoma model (2). Moreover, rVBMDMP selectively inhibited endothelial cell and human colon cancer cell proliferation, induced endothelial cell apoptosis *in vitro* and suppressed human colon cancer xenograft growth in Balb/c-nu mice (3). We determined that the interaction of rVBMDMP with αVβ3 integrin is critical for rVBMDMP binding to cells and mediates the rVBMDMP-induced inhibition of proliferation (4).

Integrins are a family of heterodimeric transmembrane proteins comprising unrelated α and β subunits that serve as receptors for extracellular matrix (ECM) proteins such as fibronectin (FN), laminins and collagens. In mammals, 18 types of α subunits and 8 types of β subunits assemble to form 24 different receptors. Integrins initiate a variety of downstream signaling events including survival or death pathways in response to ECM ligation (5). The integrin αVβ3 receptor is implicated in cardiovascular and bone function and recognizes glycoprotein ligands such as vitronectin and

FN. Upon activation of the integrin αVβ3 receptor, downstream molecules, including phosphatidylinositol 3 kinase (PI3K)/Akt, are phosphorylated, which increases cell tolerance to chemotherapy, resulting in secondary resistance in a variety of ways (6). In our previous study, we demonstrated that rVBMDMP binds αvβ3 integrins and enhances the growth inhibitory activity of cisplatin in A549 cells (7). We also found that the expression of the multidrug resistance protein 2 (MRP-2) showed a downward trend in A549 cells following treatment with rVBMDMP (unpublished data).

MRP-2 is a member of the ATP-binding cassette (ABC) transporter superfamily. ABC genes are divided into 7 distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP and GCN20) and encode proteins that transport various molecules across extracellular and intracellular membranes (8). MRP-2 is a member of the MRP subfamily, which is involved in multidrug resistance (9). Its substrates include anticancer drugs, such as vinblastine and thus MRP-2 contributes to drug resistance in mammalian cancer cells. Therefore, we speculated that the rVBMDMP-mediated inhibition of MRP-2 has the potential to reverse tumor cell resistance to chemotherapeutic drugs.

In the present study, we demonstrated that rVBMDMP inhibited cisplatin-resistant A549/DDP human lung carcinoma cell proliferation using *in vitro* and *in vivo* models of tumor growth. We also demonstrated that rVBMDMP potently reversed A549/DDP cisplatin resistance by inhibiting MRP-2 expression, which may occur via the PI3K/Akt pathway. These data suggest that rVBMDMP could be a potentially useful therapeutic molecule targeting human lung cancer.

Materials and methods

rVBMDMP. rVBMDMP (6.4 kDa) was produced in BL-21 *E. coli* using the pGEX-4T-1-VBMDMP expression plasmid and purified as previously described (1).

Cell culture. Human lung carcinoma cells (A549) and cisplatin-resistant human lung carcinoma cells (A549/DDP) were obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China) and maintained in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA), supplemented with 10% (v/v) dialyzed heat-inactivated bovine serum (BS) (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in 5% CO_2.

Cell viability assay. Cell viability was determined using the MTS assay. In brief, ~1.0x10^4 A549 and A549/DDP cells/well were plated in 96-well plates and incubated overnight. Cells were treated with various concentrations of rVBMDMP and cisplatin for 48 h, and 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTS, 5 g/l in phosphate-buffered saline (PBS)] (Promega, Madison, WI, USA) was added. The plates were incubated for 6 h, and the formed formazan dye was dissolved in 100 μl of DMSO (Sigma-Aldrich, St. Louis, MO, USA). Absorbance was recorded at 570 nm using a Biotek Synergy2 microplate reader (Biotek Instruments, Winooski, VT, USA). All experiments were repeated 3 times. Cell viability was calculated as: Cell viability rate (%) = (T - B)/(U - B) x 100%; where T is the treated cell absorbance, U is the untreated cell absorbance and B is the background absorbance when neither drug nor MTS was added.

Signal transduction antibody array. Serum-starved A549 cells were treated with 10 μmol/l rVBMDMP for 30 min, which was optimal for inhibiting endothelial cell proliferation and were lysed in 0.5% Triton X-100 buffer. This rVBMDMP concentration was determined to be optimal at inhibiting A549 cell proliferation in this study. The antibody array membrane (HM3000 signal transduction antibody array; Hypromatrix Inc., Worcester, MA, USA) was treated in blocking buffer containing 0.01% Tween-20 followed by incubation with sample diluted in 1% dry milk/PBS for 2 h at room temperature with slow shaking at 40 rpm. After the antibody filters were incubated with the supernatant protein solution at room temperature for 2 h, the antibody array filter was washed with TBST and blotted with HRP-conjugated anti-phospho-tyrosine monoclonal antibodies for 2 h. Anti-phospho-tyrosine reactivity was visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposed to X-ray film. The gray-scale chip scanogram was analyzed with chip image analysis software (QuantArray, Packard Biochip Technologies Inc. USA) to correct for protein signals. Immunoreactivity on the chip that had been incubated with control cell lysate was set to 1 for each spotted antibody. Phosphorylation ratios >2 or <0.5 were considered to indicate increased or decreased phosphorylation, respectively.

Western blot analysis. The anti-integrin αV, anti-integrin β3, anti-MRP-2, anti-NF κB, anti-caspase 3, anti-PARP, anti-bcl2, anti-Akt, anti-p-Akt, anti-PI3K, anti-pPI3K and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An anti-GAPDH antibody (Upstate Biotechnology, Lake Placid, NY, USA) was used as a loading control. After the treatments, the cells were collected and lysed. Approximately 100 ng of total protein was electrophoresed on a 10% SDS-PAGE gel and then transferred to a PVDF membrane. After blocking the membrane with 5% nonfat milk in PBS + 0.1% Tween-20 overnight at 4˚C, the blot was incubated with the primary antibody for 1 h, washed with PBS + 0.1% Tween-20 3 times (15 min each time), incubated with the secondary antibody (IgG) conjugated with horseradish peroxidase for 1 h and washed with PBS + 0.1% Tween-20 3 times. The signal was visualized with a chemiluminescence kit (SuperSignal, Pierce).

In vivo tumor growth inhibition studies. Female 6-week old Balb/c-nu mice weighing ~16 g were implanted with 2x10^6 A549/DDP human lung cancer cells into the subcutis on the back. Tumor length and width were measured using a vernier caliper, and the tumor volume was calculated using the standard formula of length x $width^2$ x 0.52 (10). When the tumors were ~100 mm^3, the animals were divided into groups of 5 mice. rVBMDMP (5 mg/kg), the angiogenesis inhibitor TNP-470 (20 mg/kg), cisplatin (10 mg/kg), a combination of rVBMDMP (5 mg/kg) and cisplatin (10 mg/kg) and vehicle control were administered via intravenous injection twice daily for 16 days. Mice were weighed twice weekly. Tumor volume was calculated every 3 days. Tumor volume ± SD was plotted vs. time over the treatment period. Upon treatment termination,

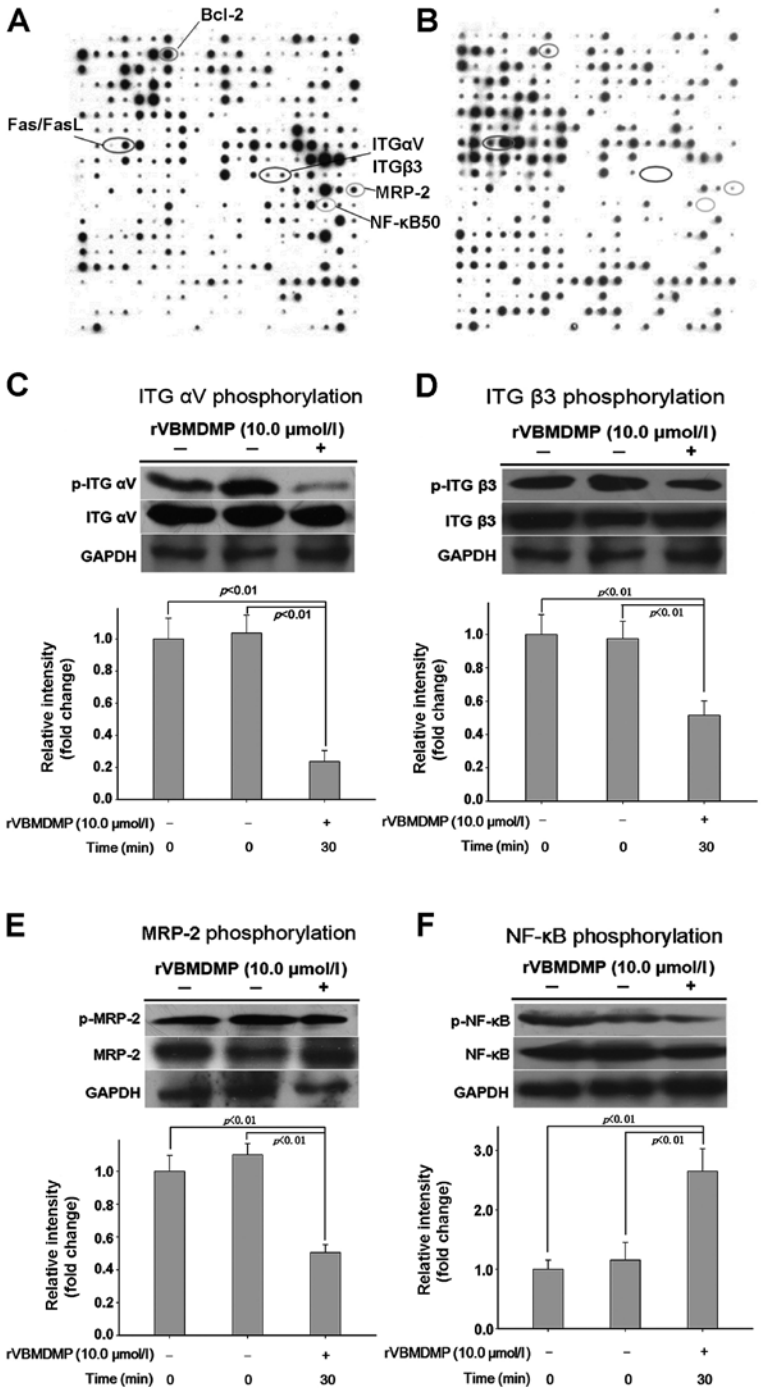

Figure 1. Signal transduction antibody array and immunobloting. (A and B) X-ray film of protein phosphorylation antibody chips: untreated controls (A) and 10 μmol/l rVBMDMP (B). (C and D) Immunoblots for phospho-integrin αV (C) and phospho-integrin β3 (D) show that 10 μmol/l rVBMDMP inhibited integrin αV and integrin β3 phosphorylation. ITG, integrin. (E and F) Phospho-MRP-2 and phospho-NF-κB immunoblots show that rVBMDMP significantly increased multidrug resistance protein 2 (MRP-2) and NF-κB phosphorylation. (C-F) Bar charts under the immunoblots show quantification of protein levels. Expression of each protein was normalized to the GAPDH level and the control was set to 1. The mean of 3 separate experiments is shown. Student's t-test was used to evaluate statistical significance (n=3). rVBMDMP, recombinant VBMDMP.

the mice were weighed and sacrificed and their tumors were excised, weighed and photographed. The mean tumor weight per group was calculated. The mean ratio of the treated tumor weight to the mean vehicle control tumor weight x 100 was subtracted from 100% to provide the tumor growth inhibition for each group. All images were captured with a Canon digital camera and developed with Kodak 400 DK-coated TMAM. The experiments were performed using 5 mice per group and all animal procedures were performed in accordance with institutional guidelines. The study protocol was approved by the Ethics Committee of Guangzhou Medical University.

Statistical analysis. Continuous data are expressed as the mean ± SD. Comparisons between groups were performed using the Student's t-test. Analysis of variance was used to examine differences in response to treatments and between groups. P-values <0.05 were considered to indicate statistically significant results.

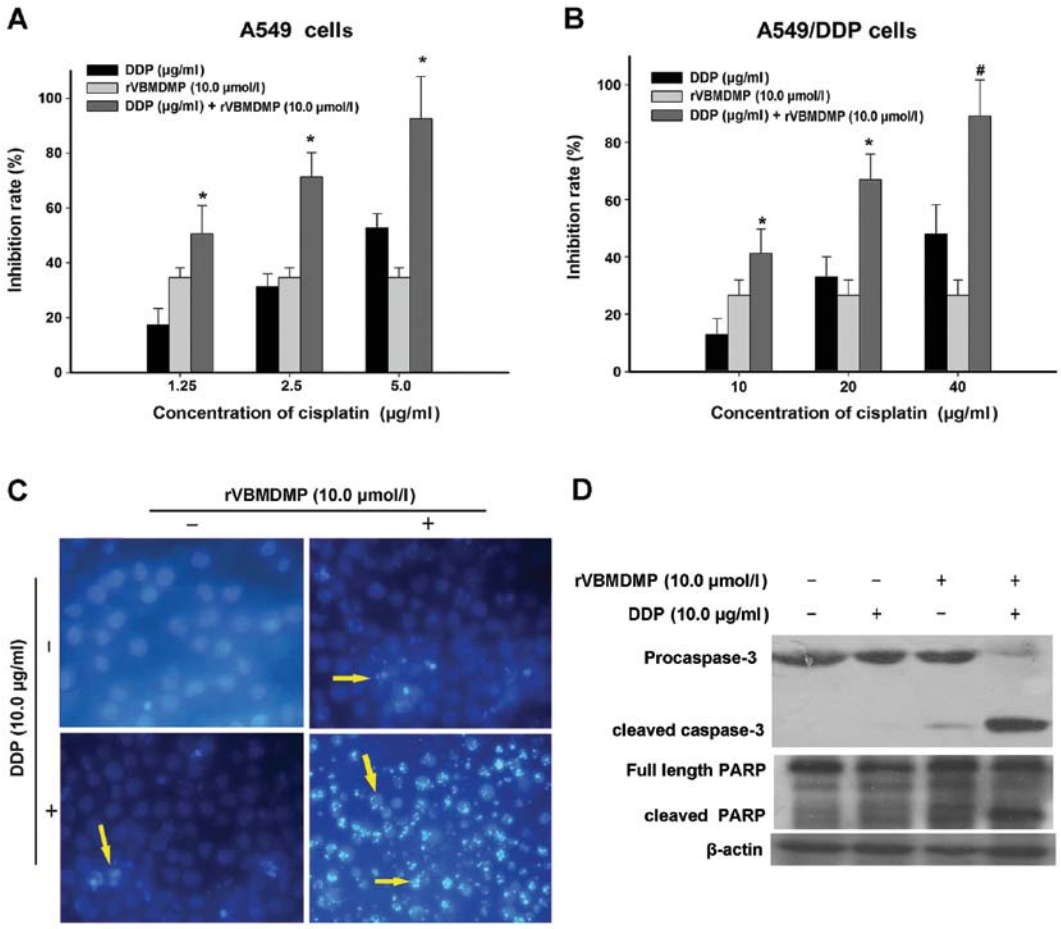

Figure 2. Effect of cisplatin combined with rVBMDMP on A549/DDP cell proliferation. (A and B) Effects of cisplatin, rVBMDMP or cisplatin combined with rVBMDMP on the proliferation of A549 (A) and A549/DDP cells (B). Values are expressed as mean ± SD (n=4). *P<0.01 vs. control (Student's t-test). (C) A549/DDP cells were cultured with RPMI-1640 with cisplatin, rVBMDMP or the combination of cisplatin and rVBMDMP as indicated for 48 h. Cell apoptosis was observed by Hoechst 33258 staining. Arrowheads indicate cells with abnormal nuclei, indicating fragmentation of nuclei/chromatin. (D) A549/DDP cells were treated as in C, collected and subjected to western blot analyses with specific antibodies directed against caspase-3, PARP and β-actin. DDP, cisplatin; rVBMDMP, recombinant VBMDMP.

Results

rVBMDMP treatment alters the phosphorylation of signaling proteins. To explore the molecular mechanism of rVBMDMP-mediated lung cancer inhibition, we examined the effects of rVBMDMP on the phosphorylation of 400 signaling proteins using a protein phosphorylation chip (Fig. 1A and B). rVBMDMP treatment increased phosphorylation (defined as a 2-fold or higher increase compared with the controls) of focal adhesion kinase (FAK), caspase-6, Fas, FasL and FAF1. rVBMDMP treatment decreased phosphorylation (defined as a 0.5-fold or more decrease compared with the controls) of integrin αV, integrin β3, PI3K/Akt, NF-κB and MRP-2 (Table I). Western blot analysis confirmed that treatment with 10 μg/ml rVBMDMP for 30 min was sufficient to inhibit integrin αV, integrin β3, MRP-2 and NF-κB phosphorylation (Fig. 1C-F). These results were concordant with the antibody microarray data.

rVBMDMP promotes A549/DDP cell cisplatin sensitivity and apoptosis. We treated A549/DDP cells with cisplatin concentrations of 5, 10, 20 and 40 μg/ml and determined that higher cisplatin concentrations inhibited A549/DDP cell proliferation. The concentrations of cisplatin required for A549/DDP cell growth inhibition were significantly higher than those required to inhibit A549 cell proliferation (Fig. 2A). After treating A549/DDP cells with 20 μg/ml cisplatin for 48 h, the growth inhibition rate was 32.9±7.1%, which increased with increasing cisplatin concentration (Fig. 2B). According to the formula $IC_{50} = lg^{-1}$ [Xm-i (P-0.5)], the IC_{50} in A549/DDP cells treated with cisplatin for 48 h was 31.19 μg/ml. The resistance index of A549/DDP cells to cisplatin was the IC_{50} of A549/DDP cells divided by IC_{50} of the A549 cells or 31.19/4.614 μg/ml=6.759. This result demonstrates that the A549/DDP cell line has a certain resistance to cisplatin, which was suitable for this drug resistance study.

Next, A549/DDP cells were treated with 10 μM rVBMDMP along with 10, 20 or 40 μg/ml cisplatin for 48 h (Fig. 2B, Table II). When A549/DDP cells were treated with 10 μg/ml cisplatin alone, the growth inhibition rate was 12.8±5.6% and the inhibitory rate increased to 45.2±8.5% when combined with 10 μM rVBMDMP. The Q value was 0.88. When A549/DDP cells were treated with 20 μg/ml cisplatin alone, the growth inhibition rate was 32.9±7.1% and the inhibitory rate increased to 66.9±8.9% when combined with 10 μM rVBMDMP. The Q value was 1.15. When A549/DDP cells were treated with

Table I. Ratios of cell signaling protein phosphorylation levels in A549 cells after 1.0 μmol/l VBMDMP treatment for 30 min.

Position	Symbol	Ratio	Description and function
1	14-3-3	3.90	Critical for cell transformation and mitotic signaling
2	c-Abl	2.44	Abelson murine leukemia virus; a 120-kDa protein with tyrosine kinase activity and an SH2 domain
42	Brk	2.31	Human homolog of Sik (Src-related intestinal kinase)
43	Brm	2.03	Similar to the brahma protein of *Drosophila*; helicase and ATPase activities
61	Caspase-6	2.30	Cysteine-aspartic acid protease 6
72	CD27	2.04	Homodimeric lymphocyte-specific surface antigen, belongs to the TNF receptor superfamily
82	Cdk2	2.66	Cyclin-dependent protein kinase
89	CIDE-B	2.39	A DNAse that is responsible for DNA degradation during apoptosis
90	Clathrin	2.12	Clathrin
102	Cyclin B	3.56	Cyclin protein B
103	Cyclin D3	3.52	Cyclin protein D3
104	Cyclin E	2.87	Cyclin protein E
109	Desmoglein	2.33	A member of the cadherin family of adhesion molecules
112	DMBT1	2.05	Deleted in malignant brain tumors 1; a candidate tumor-suppressor gene
121	E2F1	2.28	E2F transcription factor 1
122	EGFR	4.13	Epidermal growth factor receptor
123	p-EGFR	2.43	Phosphorylated epidermal growth factor receptor
124	Egr-1	3.93	EGR family of C2H2-type zinc-finger proteins, is a cancer suppressor gene
125	Egr-2	4.25	EGR family of C2H2-type zinc-finger proteins, is a cancer suppressor gene
126	Egr-3	4.52	EGR family of C2H2-type zinc-finger proteins, is a cancer suppressor gene
129	EphA4	2.56	Ephrin receptor A4
130	EphB1	5.24	Ephrin receptor B1
131	Eps8	3.65	Epidermal growth factor receptor substrate 8
141	FAF-1	6.64	FAS interacting protein
142	FAK	5.50	Focal adhesion associated protein-tyrosine kinase
143	Fas	5.08	A member of the tumor necrosis factor family of cell surface receptors
144	FasL	2.27	Fas ligand
146	FGFR1	7.32	Fibroblast growth factor receptor 1
147	FGFR2	2.70	Fibroblast growth factor receptor 2
148	FGFR3	2.11	Fibroblast growth factor receptor 3
149	FGFR4	3.23	Fibroblast growth factor receptor 4
150	FHIT	5.25	A member of the histidine triad protein family; a candidate tumor suppressor
161	GATA-1	4.05	Transcription factor
162	GATA-2	4.96	Transcription factor
163	GATA-3	7.17	Transcription factor
164	G-CSFR	2.12	Colony stimulating factor receptor
165	gp130	2.30	Glycoprotein 130
166	Granzyme B	4.50	Cytotoxic T-lymphocyte-associated serine esterase 1
167	GRB2	2.27	Growth factor receptor-bound protein 2
169	GRB14	3.56	Growth factor receptor-bound protein 14
170	GRK2	4.44	G protein-coupled receptor kinase 2
182	IFN-aRa	2.82	Type I interferon α receptor α
183	IFN-gRa	7.28	Type II interferon γ receptor α
184	IL1R1	4.64	Interleukin-1 receptor 1
186	IL2Rβ	5.43	Interleukin-2 receptor β
187	IL2Rγ	3.77	Interleukin-2 receptor γ
188	IL3	2.86	Interleukin-3
189	IL4Ra	2.06	Interleukin-4 receptor α
202	Jak1	2.65	Janus kinase 1
203	Jak2	2.49	Janus kinase 2

Table I. Continued.

Position	Symbol	Ratio	Description and function
206	p-JNK1,2,3	2.13	Phosphorylated c-Jun N-terminal kinases 1,2,3
209	KAP	2.18	A dual specificity phosphatase that interacts with cyclin-dependent kinases
222	MEK1	2.08	Mitogen-activated protein kinase kinase 1
252	Nip3	2.03	A member of the BCL2/adenovirus E1B 19 kDa-interacting protein (BNIP) family, Nip3 preferentially binds to Bcl-xL and induces apoptosis by suppressing the anti-apoptosis activity of Bcl-xL
282	PDGFRβ	2.39	Platelet-derived growth factor receptor β
291	PTEN	2.06	Phosphatase and tensin homolog; the PTEN gene is a tumor suppressor gene
292	SH-PTP	2.33	SH-protein tyrosine phosphatase 1
307	RalA	2.46	Small GTPase superfamily; Ras family of proteins
308	RanBP-1	2.31	Ras-related nuclear protein BP-1
311	RARr	2.18	Retinoic acid receptors
312	RXR a,b, r	2.30	Retinoid X receptors a, b, r
325	RIP	2.39	Receptor interacting protein
331	P-Selectin	3.12	Cell adhesion molecule
332	SHC	2.56	Src homology 2 domain containing
343	Blk	2.27	Proto-oncogenic non-receptor tyrosine kinase
346	Lck	2.72	Leukocyte-specific protein tyrosine kinase
347	Lyn	3.54	A member of the Src family of protein tyrosine kinases
351	STAM	3.59	Signal transducing adaptor molecule
364	TANK	2.68	TRAF-associated NF-κB activator
365	TCRα	3.92	T-cell receptor α
366	TCRβ	4.14	T-cell receptor β
367	TDAG51	3.32	T-cell death associated gene 51
370	Thyroid Rα1	3.56	Thyroid hormone nuclear receptor α 1
371	TIA-1	5.52	A member of an RNA-binding protein family; a mediator of apoptotic cell death
372	TIAR	4.06	TIA receptor
375	TOSO	2.57	
392	VDR	2.99	Vitamin D receptor
394	VEGFR2	2.35	VEGF receptor 2
397	XRCC4	2.38	X-ray repair cross-complementing protein 4
14	APC	0.43	Adhesion protein
27	Bcl-2	0.42	B-cell lymphoma 2
39	BARD1	0.36	BRCA1-associated RING domain gene 1 is a major cellular binding partner of BRCA1
40	BRCA1	0.42	Breast cancer 1
60	Caspase-5	0.49	Cysteine-aspartic acid protease 5
100	CUL-1	0.43	A member of the cullin protein family
118	DR5	0.32	Death receptor 5
139	Ezrin	0.48	Cytoplasmic protein; a major cytoplasmic substrate of various protein-tyrosine kinases
158	GADD45	0.30	Growth arrest and DNA damage 45
174	Ne-dlg	0.49	Neuronal and endocrine dlg (Discs large)
175	hIL	0.50	IAP family member
179	ICSBP	0.49	Interferon consensus sequence-binding protein
180	ID1	0.38	A member of the Id family of basic helix-loop-helix (bHLH) proteins
194	ITG αV	0.12	Integrin α subunit
195	ITG β1	0.12	Integrin β subunit (CD29)
196	ITG β3	0.14	Integrin β subunit
197	IRAK	0.25	IL-1 receptor-associated kinase
198	IRF1	0.41	Interferon regulatory factor-1
213	LIFR	0.41	Leukemia inhibitory factor receptor

Table I. Continued.

Position	Symbol	Ratio	Description and function
215	MAD2	0.16	Mitotic arrest-deficient 2
216	Maspin	0.12	A serpin and tumor suppressor gene
217	Max	0.45	Transcription factor
218	MDA-7	0.40	Melanoma differentiation-associated protein-7
220	MRP-2	0.47	Multiple drug resistance protein
235	NF1GRP	0.27	Neurofibromin protein
237	NFATC	0.46	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent
238	NF-κB-p50	0.40	Nuclear factor-κB 50
256	Notch	0.46	A human gene encoding a single-pass transmembrane receptor
278	Pax-5	0.48	Nuclear transcription factor

Table II. Inhibition rate and Q values of cisplatin in combination with rVBMDMP on A549/DDP cell growth.

Group	Growth inhibition rate (%)	Q value	Cisplatin IC_{50}	Reversal index (RI)
DDP (10 μg/ml)	12.8±5.6		31.19	
DDP (20 μg/ml)	32.9±7.1			
DDP (40 μg/ml)	52.0±10.2			
DDP (10 μg/ml) + rVBMDMP (10.0 μM)	45.2±8.5	0.88	11.82	2.639
DDP (20 μg/ml) + rVBMDMP (10.0 μM)	66.9±8.9	1.15		
DDP (40 μg/ml) + rVBMDMP (10.0 μM)	89.1±12.3	1.22		

A Q value of 0.85-1.15 represents the summation effects of the two drugs; a Q value >1.15 indicates a synergistic effect of the two drugs; a Q value <0.85 indicates an antagonistic effect of the two drugs. rVBMDMP, recombinant VBMDMP.

40 μg/ml cisplatin alone, the growth inhibition rate was 52±10.2% and the inhibitory rate increased to 89.1±12.3% when combined with 10 μM rVBMDMP. The Q value was 1.22. According to the formula $IC_{50} = lg^{-1}$ [Xm-i (P-0.5)], the IC_{50} was 11.82 μg/ml when combined with 10 μM rVBMDMP in A549/DDP cells. Thus, the multidrug resistance reversal index (RI) was 2.639. Together, these results demonstrated that the combined application of rVBMDMP with cisplatin produced an additive inhibition to significantly reduce A549/DDP cell survival.

We next evaluated A549/DDP cell apoptosis after cisplatin and rVBMDMP treatment using Hoechst 33258 staining (Fig. 2C). Apoptotic cells were observed after treatment with 10 μM rVBMDMP or 10 μg/ml cisplatin alone for 48 h. However, the combination of these 2 drugs markedly enhanced A549/DDP cell apoptosis compared with the control group, which underwent little apoptosis. The nuclei as observed by normal fluorescence microscopy were large and evenly stained; pyknotic nuclei appeared smaller and the nuclear chromatin was densely stained towards the edge or showed chunky dense staining in apoptotic cells.

As shown in Fig. 2D, caspase-3 was mildly activated (cleaved) in the A549/DDP cells following treatment with rVBMDMP alone and was nearly completely cleaved when combined with cisplatin. PARP cleavage showed a similar trend. This suggests that combined treatment causes caspase-3 activation, thereby inducing apoptosis and PARP cleavage. This may be coupled with MRP-2 downregulation, which then blocks cisplatin cellular efflux. This may be one of the mechanisms involved in the reversal of A549/DDP cell chemotherapeutic resistance by rVBMDMP.

rVBMDMP and cisplatin treatment in combination significantly inhibit survival. We next investigated the molecular mechanism by which the combination treatment of rVBMDMP and cisplatin mediates anti-survival effects in A549/DDP cells.

After treatment with 10 μM rVBMDMP alone, A549/DDP cells showed downregulation of MRP-2, integrin αV and NF-κB p50 protein expression, while cisplatin alone had no effect on MRP-2, Bcl-2, integrin αV or NF-κB p50 protein expression (Fig. 3A). However, upon the combination treatment of cisplatin and rVBMDMP, levels of the above proteins were significantly reduced compared with the controls. rVBMDMP downregulation of MRP-2, integrin αV and NF-κB p50 protein expression may be related to the reversal of A549/DDP cell drug resistance.

Total PI3K and Akt protein levels were not altered in all 4 A549/DDP cell treatment groups, while PI3K/Akt protein phosphorylation was markedly decreased following 10 μM rVBMDMP treatment, indicating that rVBMDMP

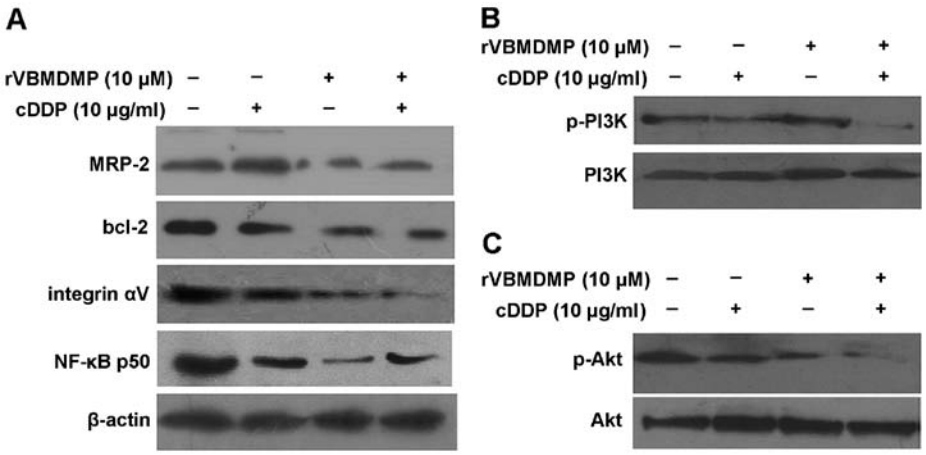

Figure 3. Combination of cisplatin and rVBMDMP significantly inhibits A549/DDP cell survival signal. A549/DDP cells were cultured in RPMI-1640 with cisplatin, rVBMDMP, or the combination of cisplatin and rVBMDMP as indicated for 48 h. (A) Cells were collected and subjected to western blot analyses with specific antibodies against multidrug resistance protein 2 (MRP-2), Bcl-2, integrin αV, NF-κB (P50) and β-actin. (B and C) Western blot analysis of cell treated as in A for phosphatidylinositol 3 kinase (PI3K) and Akt protein expression and phosphorylation in cultured A549/DDP cells. DDP, cisplatin; rVBMDMP, recombinant VBMDMP.

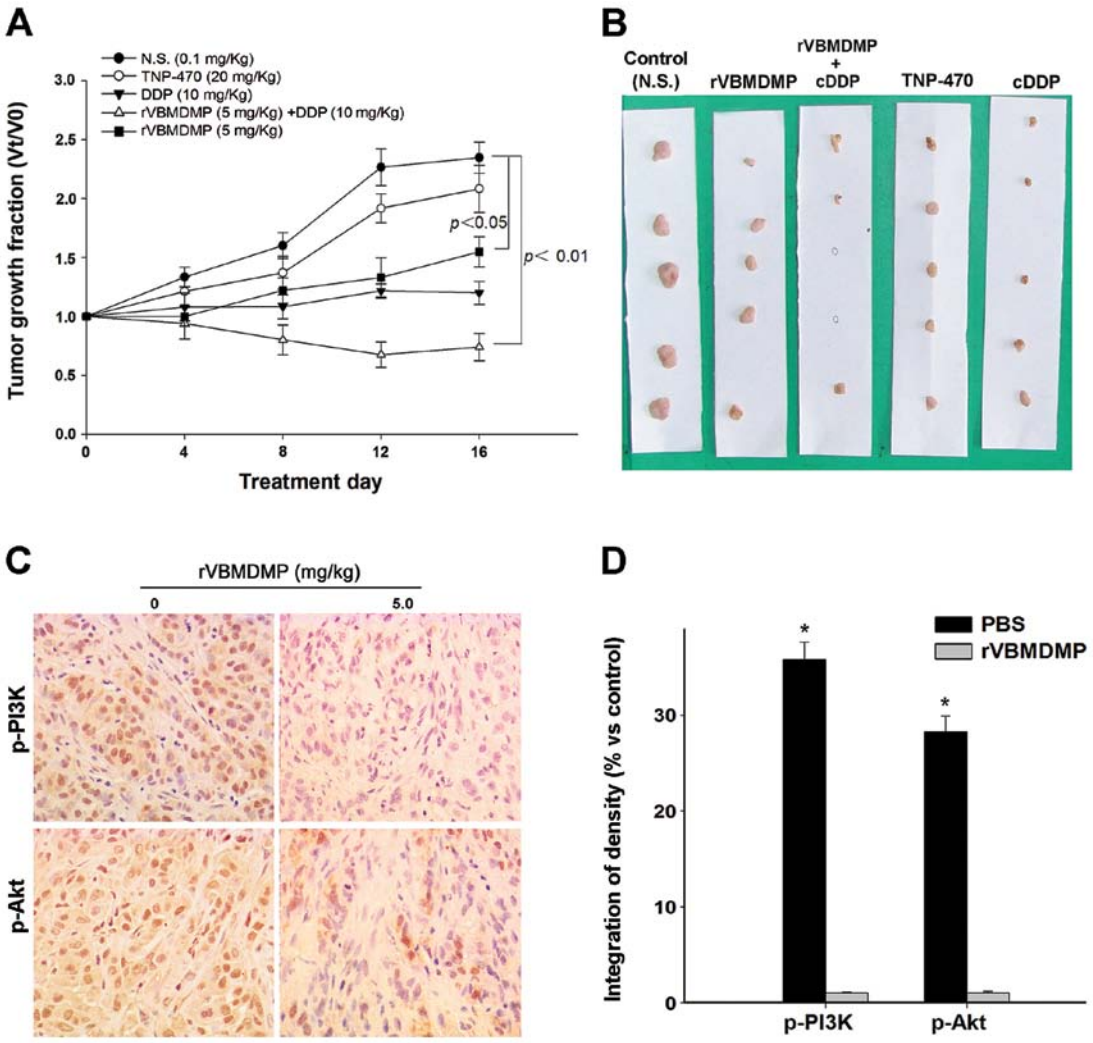

Figure 4. Effects of rVBMDMP on primary human lung carcinoma xenograft models in nude mice. (A) Growth inhibition of A549/DDP human lung carcinoma xenografts treated as indicated. Cells ($2x10^6$) were injected subcutaneously into 4- to 5-week-old female athymic nude mice. rVBMDMP (5 mg/kg) alone or in combination with cisplatin (10 mg/kg) was injected intravenously twice daily for 16 days. The control group received the saline vehicle. TNP-470, an angiogenesis inhibitor, and cisplatin were used as positive controls. The tumors were allowed to grow to ~100 mm^3. Each point represents the mean ± SD of 5 mice per group. (B) Image of solid tumors obtained after 16 days of treatment. (C) Immunohistochemistry of phosphorylated phosphatidylinositol 3 kinase (PI3K) and phosphorylated Akt in endpoint tumors in the rVBMDMP (5 mg/kg)-treated and control groups. (D) Integrated density measurements for p-pI3K and p-Akt shown as mean ± SD. *P<0.01 vs. control (n=5 fields/group). DPP, cisplatin; rVBMDMP, recombinant VBMDMP.

inhibited PI3K and Akt phosphorylation in the A549/DDP cells (Fig. 3B and C). Phosphorylated PI3K/Akt levels were not altered in the cisplatin-treated group. These data suggest that the PI3K/Akt signal transduction pathway may be associated with the rVBMDMP-mediated reversal of multidrug resistance.

rVBMDMP-mediated human lung carcinoma xenograft growth inhibition in BALB/c nude mice. To investigate the inhibition of rVBMDMP on A549/DDP cell growth *in vivo*, we examined the effects of rVBMDMP on established primary human lung carcinoma xenograft models in nude mice. Our results showed that rVBMDMP significantly inhibited human lung carcinoma xenograft growth (Fig. 4). rVBMDMP treatment decreased the tumor growth rate as evaluated by measurement of the tumor volume at regular intervals (Fig. 4A). Administration of 5 mg/kg rVBMDMP combined with 10 mg/kg cisplatin resulted in 37 and 74% reduction in tumor volume, respectively (Fig. 4B). After 16 days of treatment, the final wet tumor weight in the 5 mg/kg rVBMDMP-treated group was reduced by 77% ($P<0.05$), whereas the tumor weight was reduced by 4% after TNP 470 (20 mg/kg) treatment and 42% after cisplatin (10 mg/kg) treatment.

Discussion

Our previous research determined that rVBMDMP and the tumstatin 197-215 amino acid peptide can significantly inhibit tumor cell (A549 and SW480 cells) proliferation and growth in a dose-dependent manner, (3,7) while there were no significant effects on normal human embryo lung (KMB-17) and Chinese hamster ovary (CHO-K1) cell proliferation and growth. These results indicate that rVBMDMP not only preserves the anti-tumor activity of the tumstatin 197-215 amino acid peptide but also has relative selectivity to cancer cells compared with normal cell lines. rVBMDMP significantly inhibited human lung and colon cancer xenograft growth in nude mice in a dose-dependent manner (3). Therefore, the tumstatin 197-215 amino acid peptide as part of rVBMDMP may be responsible for its inhibition of tumor cell proliferation and growth. Shahan *et al* confirmed that the tumstatin N-terminal 197-215 peptide exhibits biological function by binding integrin αVβ3 on the tumor cell surface (11). However, the underlying detailed mechanism is not clear. Our previous study confirmed that rVBMDMP also binds integrin αVβ3 (4).

Integrin αVβ3 expression is highly cell specific, with nearly no expression on the surface of resting endothelial cells and some normal cells, such as hepatic stellate cells (12). However, αVβ3 expression is significantly higher in lung adenocarcinoma A549 cells (13), prostate cancer (14) and breast cancer (15). Integrin has a bidirectional signal transmission function: its outward intracellular signal transduction regulates cell adhesion and migration, while integrin ligand binding triggers signals to regulate cell growth, differentiation and apoptosis (16,17). In the present study, the antibody array results revealed that rVBMDMP treatment can down-regulate integrin αVβ3 subunit phosphorylation in A549 cells, suggesting effects on its downstream pleiotropic and complex signal transduction.

Integrin signal transduction is closely related to FAK activation (18,19). FAK is a cytoplasmic non-receptor tyrosine kinase and its activation is accompanied by the accumulation of focal adhesion. The FAK signal transduction pathway can be activated substantially by integrins. Activated FAK binds multiple intracellular proteins that contain SH2 domains, thus activating several signaling pathways. Among these is the PI3K/Akt pathway, on which we previously focused our studies (20). PI3K regulates signaling pathways that are involved in multiple cellular functions including survival, proliferation, apoptosis, cell differentiation and cytoskeleton structure. PI3K is a phosphoinositide-dependent kinase family member that specifically catalyzes PI-4,5-P2 and Ptdlns-4-P to generate Ptdlns-3, 4, 5-P3 and Ptdlns-3, 4-P2, respectively. The last 2 multi-phosphatidylinositol derivatives have biological roles as messenger molecules by binding and activating Akt, thus causing tumor cell proliferation and inhibiting apoptosis (21), which is an important cause of tumor drug resistance (22). Thus, the PI3K pathway may play a role in multidrug resistance (23). FAK can recruit and directly activate PI3K, which activates its downstream target Akt.

Akt is a main target of PI3K and is closely related to a variety of cell biological behaviors such as metabolism regulation, cell survival and particularly apoptosis (24,25). Activation of key survival signaling molecules such as PI3K/Akt, especially increasing Akt phosphorylation levels, is not only closely related to cancer cell development and apoptosis inhibition but is also a main step leading to multidrug resistance (26,27). Activated Akt can promote cell growth and proliferation by phosphorylating downstream molecules such as mammalian target of rapamycin (mTOR), $p27^{WAF1/Cipl}$, GSK3 and tuberous sclerosis complex 2 (TSC2) (28,29). It also inhibits apoptosis via NF-κB and 14-3-3 phosphorylation-mediated down-regulation of FasL-induced apoptosis protein (30,31) as well as phosphorylation of several apoptosis-related molecules including Bcl-2 family members such as Bcl-2, Bcl-xL and Bcl-xs (32,33), inhibitor of apoptosis protein family members (IAPs) (34) and caspase-8, -9 and -3 (35), which inhibit apoptosis, thus inducing cancer cell chemotherapeutic drug resistance (36). Our results demonstrated that rVBMDMP treatment of lung cancer cells also affected integrin-FAK pathway signal transduction by downregulating FAK, PI3K, Akt and NF-κB survival signaling molecule phosphorylation and further affecting A549/DDP lung cancer survival cell signaling, weakening its cell survival ability and even directly inducing apoptosis.

Here, we determined that rVBMDMP treatment when combined with cisplatin can reverse A549/DDP cell multidrug resistance. This result was displayed by i) a significantly decreased cisplatin IC_{50} in A549/DDP cells and (2) significantly decreased MRP expression in A549/DDP cells. The results obtained from the animal experiments also demonstrated that rVBMDMP treatment combined with cisplatin can effectively inhibit A549/DDP cell growth in nude mice. These data suggest that rVBMDMP is not only an effective antitumor drug, but it can also reverse the resistance of A549/DDP cells to cisplatin.

Chemotherapy resistance is a major cause of non-small cell lung carcinoma (NSCLC) chemotherapy failure and disease progression, and chemotherapy tolerance-induced tumor cell apoptosis is an important mechanism of tumor resistance. Cisplatin is a commonly used drug with a high curative effect

on lung cancer. Cisplatin resistance is often indicative of multidrug resistance, the phenomenon in which cells exhibit insensitivity to many types of chemotherapy drugs. Therefore, clinical follow-up treatment for patients with cisplatin resistance is often difficult.

Multidrug resistance consists of a complex mechanism, in which MRP-2 plays a major role. MRP-2, also called multispecific organic anion transporter (cMOAT), functions as a transport protein for organic anions and a variety of drugs (37). MRP-2 is considered as the mediator of cisplatin resistance, as neither P-gp nor MRP1, related multidrug resistance proteins, recognize cisplatin as substrate. Ishikawa *et al* first demonstrated that the MRP-2/GS-X pump could transport glutathione-cisplatin conjugates from the cells, which mediates tumor cell resistance to cisplatin (38). The authors determined that the glutathione S efflux pump activity in tumor cells with high MRP-2 expression was enhanced, suggesting that MRP-2 can identify and transport glutathione-drug conjugates and promote tumor drug or modified product efflux to produce multidrug resistance (39). The present study also observed downregulation of PI3K/Akt phosphorylation in human lung cancer A549/DDP cells following rVBMDMP treatment, weakening survival signaling. Expression of anti-apoptotic proteins Bcl-2 and MRP-2 were also reduced, thus weakening the anti-apoptotic ability and the drug pumping effect of cisplatin-resistant cells. It has been suggested that rVBMDMP can weaken A549/DDP cell tolerance to cisplatin, enhance cisplatin sensitivity, facilitate endogenous cisplatin-induced apoptosis signals and even reverse the drug resistance traits of A549/DDP cells. Above are some of the molecular mechanisms by which rVBMDMP increases chemotherapy sensitivity and reverses the effects of multidrug resistance. These results suggest that the antitumor activity of rVBMDMP on A549 lung cancer cells was not related to A549/DDP cell drug resistance, indicating there is no cross resistance to cisplatin and rVBMDMP. Conversely, these results also suggest that cisplatin and rVBMDMP affect different pathways. Therefore, rVBMDMP treatment can still have favorable effects for patients suffering from cisplatin-resistant lung adenocarcinoma.

In conclusion, the results of this study provide a theoretical and experimental basis for further evaluation of the molecular mechanisms of rVBMDMP in regulating tumor cell signaling networks and reversing drug resistance in lung cancer.

Acknowledgements

This study was supported by the Natural Science Foundation of Guangdong Province (grant no. S2012010008995) and the Doctoral Fund of the Education Ministry of China (grant no. 20124423110003.

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