

Esophageal Cancer

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LIP-rMDVA+VRL Composed of Liposomal cDNA Encoding Recombinant Multimodular Proteins rMDVA Comprising Disintegrin/Cysteine-Rich Disulfide Bond 2RGD, and C-Terminal Domain (Ammodystatin), Metalloprotease-Domain (Ammodylsin), and Dimeric Disintegrin/MLD-VGD Domain (VADD) Isolated From Vipera Ammodytes, Exerted Synergistic Action with Vinorelbine Tartrate Against Metastatic Esophageal Squamous Cell Cancer, Circumventing Chemotherapy-Mediated Systemic Toxicity

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Background: Vipera ammodytes is the most venomous solenoglyph reptile in Europe. In this study, we investigated if components of its venom proteome can exert synergistic action with conventional chemotherapy against metastatic esophageal squamous cell carcinoma (ESCC), a malignancy with rising incidence in Western countries. ESCC patients who receive conventional treatments face a poor prognosis; furthermore, standard chemotherapy is associated with stimulation of angiogenic and lymphangiogenic growth factors that may enhance the metastatic process.

Materials and Methods: We freeze dried venom from Vipera ammodytes snakes. The venom was obtained with electrodes (2-5 watts for 1-2 sec). The venom proteome was analyzed with FPLC, LC/MS/MS, 2-D electrophoresis, sequence analysis, cation exchange sulfopropyl HPLC, DEAE anion exchange HPLC, superfine size-exclusion chromatography, SDS-PAGE, HPLC gel-filtration, reverse-phase HPLC, x-ray crystallography, ¹H-NMR spectroscopy, NH₂-terminal amino acid sequencing, and microarray analysis. We have developed a large-scale expression system using genetic engineering for mass production of multimodular disintegrin (rMDVA) comprising three functional domains with recombinant DNA technology. We isolated the gene for producing MDVA, which was recombined with a bacterial DNA plasmid. Many plasmids with the MDVA gene are inserted into many bacterial cells producing rMDVA protein molecules, which are purified. This novel

recombinant MDVA (rMDVA) was encapsulated in the hydrophilic phase of liposomes with an average size of 100 μ m. Vinorelbine tartrate was encapsulated in the liposomal hydrophobic phase. The liposomal formulation with rMDVA + vinorelbine (VRL) was termed LIP-rMDVA + VRL. The in-vivo studies with human metastatic ESCC cells obtained from surgical specimens were performed in an orthotopic xenograft model in 12-week-old nude mice. After 10 days of ESCC cells implantation, IV liposomal delivery of rMDVA+VRL was administered to mice once daily. Five weeks later, mice were sacrificed for examination with immunostains for IHC (CD31, CD144, vWF, CD34, UEA-1, FvIIIrArg, bcl2L12, bcl-2/bcl-xL, bcl-w, Mcl-1, A1, K-Ras, c-Raf, MAPK, PI3K/AKT, ERK, PDGF, VEGF-C/LYVE-1/VEGFR-3, VEGF-D, VEGF-A, VEGFR1/Flt, VEGFR2/Flk, neuropilin-1/NRP1-2, cyclinD1, integrinB5, PLGF, MMP-1, MMP-2, MMP-3, MMP-9, α -SMA, typeVI collagen α 1- α 2, XVIII collagen, IV collagen, type I collagen, and autocrine motility factor/AMF). NB, WB, TEM, RT-PCR, microarray analysis, apoptotic assays (Annexin V/PI, DNA fragmentation, TUNEL, PARP, caspases 3), LDH release, MTT, BrdU, MVD, Matrigel TM of capillary endothelial cells, ELISA (IL-10, IL-12), HUVEC, and HMVEC proliferation from complex tubular networks were also assessed.

Results: The venom proteome of *Vipera ammodytes* contained 140 proteins. We isolated the multimodular disintegrin displaying cysteine-rich disulfide bond 2RGD, and C-terminal motif (ammodystatin), the dimeric MLD/VGD motif (VADD), and metalloprotease-domain (ammodylisin). The molecular identity of recombinant MDVA with the native protein isolated from snake venom was confirmed with NH₂-terminal amino acid sequencing and mass spectrometry. The liposomes leaked out of the new blood vessels exploited by the tumor delivering their chemoprotein cargo into the cancer cells after forming depots in the tumor site, where lipases, and extracellular enzymes derived from stromal cells degrade liposomal structure. The ammodystatin with the 2(ARG-GLY-ASP)RGD motifs, one in each chain which contains 65 amino acids, interacts with integrins α v β 5, α 5 β 1 exerting dual action, which inhibits adhesion of tumor cells to the extracellular matrix (ECM), blocking invasion. Activated fibroblast state of the primary stromal cell type, and ECM proteins that promote invasion of ESCC cells, and stromagenesis were inhibited according to reduced Matrigel TM coated plates, and downregulated ECM-degrading proteinases, collagens, and

MMPs. Inactivation of myofibroblast state of carcinoma-associated fibroblast (CAF), defined by inhibition of α -smooth muscle actin (α -SMA) blocked tumor progression, angiogenic, and lymphangiogenic response. Transcriptional activation of VEGF and VEGFR by chemotherapeutic adaptive cell stress was inhibited. Also, tumor endothelial cell (TEC)-induced angiogenesis was inhibited according to downregulation of VEGF progressive autocrine and paracrine signaling, VEGF-A, VEGFR1/Flt, VEGFR2/Flk, neuropilin-1/NRP1,2. Ammodystatin blocked the bFGF-induced α v β 3 integrin, an ECM protein receptor and a marker of angiogenic blood vessels, which promotes survival of endothelial cells. Also, it inhibits growth and differentiation of blood vessels by blocking vascular cell integrin α v β 5, leading to VEGF downregulation. There was inhibition of lymphangiogenesis, and lymphatic metastasis by inactivation of VEGFR3, which is expressed on lymphatic endothelial cells. Also, there was inhibition of VEGF-C stimulated cell proliferation, downregulation of LYVE1-VEGFR, and reduction of expression of mRNA-encoding cyclinD1, integrin β 5, and AMF. Furthermore, ammodystatin inhibited prenylation of Ras/MAPK signaling pathway and downstream Ras effectors (c-Raf, ERK1/2 kinase, MEK1, PI3K/AKT, PDGF/RTK), inhibiting tumoricidal cell proliferation, migration, survival, and gene transcription. Ammodystatin's downregulation of overexpressed Ras due to mutation in codon 12 GAT-GTT caused further inhibition of the dual action of VEGF blocking angiogenic factor (angiogenesis), and vascular survival factor (vasculogenesis). It also blocked endothelial cell secretion of gelatinaseA, gelatinaseB, and urokinase-type plasminogen activator with its receptor inhibiting proteolysis, and dissolution of ECM to proteins, and polysaccharides, with subsequent blockage of invasion, and migration of endothelial cells into the surrounding tissues, making impossible the formation of a network of blood vessels to nourish the tumor. VRL inhibited β -tubulin depolymerizing cytoskeleton microtubules, which led to G2/M cell cycle arrest acting synergistically with the cytostatic action of ammodystatin, which disrupted the actin cytoskeleton of tumor cells, and inhibited the α -SM actin of carcinoma-associated fibroblasts (CAF), blocking DNA replicative cycle at G1 phase, and inducing type I PCD/apoptosis after vinorelbine's phosphorylation, and downregulation of antiapoptotic genes (bcl-2/bcl-xL, bcl-w, mcl-1, A1, and bcl2L12 which is between the IRF3 and RRAS oncogenes), inhibiting cell motility and invasion. The synergistic apoptotic action of ammodystatin and VRL eradicated tumor cell masses up to 2

mm. Beyond that size, ammodystatin inhibited the development of the required vascular network. The dimeric disintegrin VADD blocked adhesion of $\alpha 4\beta 1$ integrin to vascular cell adhesion molecule1 (VCAM1), inhibiting tumor-derived neovascularization. Thus, by inhibiting αv integrins, we disrupt tumor angiogenesis. Also, VADD causes the burring phenomenon and causes destructive morphologic changes in already-formed tumoral pre-existing blood vessels. Ammodylisin induces type I PCD or apoptosis in the endothelial cells of the tumor-derived neo-vascularization. Biochemical assays LDH, BrdU, and MTT exhibited inhibition of tumor and endothelial cell proliferation, indicating cytostatic activity. Transmission electron microscopic analysis exhibited apoptotic signs of irreversible D2 stage in tumor and endothelial cells. Inhibition of tumor vessel angiogenesis after inhibition of VEGF (Flt-1, Flk-1), bFGF receptors, and αv integrins in endothelial cells of blood vessels was exhibited by HUVEC/HMVEC proliferation, MVD and IHC. Finally, there was inhibition of IL-10 and IL-12 levels indicating elimination of chemotherapy-associated hematologic toxicity (granulocytopenia), and gastrointestinal toxicity (dysphagia, mucositis), and inactivation of angiogenic growth factor production, reflecting the adaptive stress response by which ESCC cells attempt to protect themselves from chemotherapy.

Conclusion: Novel liposomal formulation LIP-rMDVA+VRL, composed of recombinant multimodular disintegrin with three functional domains, comprises an effective angiostatic, lymphangiostatic, and cytostatic agent, which exerts synergistic action with vinorelbine tartrate against metastatic ESCC, circumventing chemotherapy-mediated systemic toxicity.