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Cell-Specific Delivery of Diverse Cargos by Bacteriophage MS2 Virus-Like Particles

Carlee E. Ashley[a,†], **Eric C. Carnes**[b], **Genevieve K. Phillips**[c], **Paul N. Durfee**[f], **Mekensey D. Buley**[d], **Christopher A. Lino**[f], **David P. Padilla**[a], **Brandy Phillips**[c], **Mark B. Carter**[c], **Cheryl. L. Willman**[c,e], **C. Jeffrey Brinker**[a,b,c,f,g], **Jerri do Carmo Caldeira**[f], **Bryce Chackerian**[c,f], **Walker Wharton**[c,e], and **David S. Peabody**[c,f]

[a] Center for Micro-Engineered Materials, the University of New Mexico, Albuquerque, NM 87131, USA

[b] Department of Chemical and Nuclear Engineering, the University of New Mexico, Albuquerque, NM 87131, USA

[c] Cancer Research and Treatment Center, the University of New Mexico, Albuquerque, NM 87131, USA

[d] Chemical, Biological, and Materials Engineering, University of Oklahoma, Norman, OK 73109, USA

[e] School of Medicine, Department of Pathology, the University of New Mexico, Albuquerque, NM 87131, USA

[f] Department of Molecular Genetics and Microbiology, the University of New Mexico, Albuquerque, NM 87131, USA

[g] Self-Assembled Materials Department, Sandia National Laboratories, Albuquerque, NM 87185-1349, USA

Abstract

Virus-like particles (VLPs) of bacteriophage MS2 possess numerous features that make them well-suited for use in targeted delivery of therapeutic and imaging agents. MS2 VLPs can be rapidly produced in large quantities using *in vivo* or *in vitro* synthesis techniques. Their capsids can be modified in precise locations *via* genetic insertion or chemical conjugation, facilitating the multivalent display of targeting ligands. MS2 VLPs also self-assemble in the presence of nucleic acids to specifically encapsidate siRNA and RNA-modified cargos. Here we report the use of MS2 VLPs to selectively deliver nanoparticles, chemotherapeutic drugs, siRNA cocktails, and protein toxins to human hepatocellular carcinoma (HCC). MS2 VLPs modified with a peptide (SP94) that binds HCC exhibit a 10^4-fold higher avidity for HCC than for hepatocytes, endothelial cells, monocytes, or lymphocytes and can deliver high concentrations of encapsidated cargo to the cytosol of HCC cells. SP94-targeted VLPs loaded with doxorubicin, cisplatin, and 5-fluorouracil selectively kill the HCC cell line, Hep3B, at drug concentrations < 1 nM, while SP94-targeted VLPs that encapsidate a siRNA cocktail, which silences expression of cyclin family members, induce growth arrest and apoptosis of Hep3B at siRNA concentrations < 150 pM. Impressively,

Corresponding Authors: Carlee E. Ashley (ceashle@sandia.gov) and David S. Peabody (dpeabody@salud.unm.edu).
[†]Current Appointment: Harry S. Truman Post-Doctoral Fellow; Biotechnology and Bioengineering Department, Sandia National Laboratories, Livermore, CA 94551, USA.

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MS2 VLPs, when loaded with ricin toxin A-chain (RTA) and modified to co-display the SP94 targeting peptide and a histidine-rich fusogenic peptide (H5WYG) that promotes endosomal escape, kill nearly 100% of Hep3B cells (1×10^6 cells/mL population) at an RTA concentration of 100 fM without affecting the viability of control cells. Our results demonstrate that MS2 VLPs, due to their tolerance of multivalent peptide display and their ability to specifically encapsidate a variety of disparate cargos, induce selective cytotoxicity of cancer *in vitro* and represent a significant improvement in the characteristics of VLP-based delivery systems.

Keywords

virus-like particles; multivalent peptide display; targeted drug delivery; cancer; nanoparticle; nanocarrier

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Despite numerous advances over the past several decades, effective cancer chemotherapy remains challenging. The generalized toxicity of many drugs makes it difficult to achieve therapeutic concentrations without severe systemic side effects. Nanocarriers are being developed that address these issues by selectively delivering drugs to tumor sites. Some designs take advantage of the enhanced permeability of vasculature in the tumor vicinity to promote passive accumulation of drug nanocarriers that are too large to otherwise leave the circulation. Others seek to endow a nanocarrier with the added ability to specifically bind receptor molecules differentially expressed on the surfaces of tumor cells, in the hope that the particle will be internalized and release its contents in a cell-specific manner.

The use of nanoparticles in passive and targeted drug delivery has a relatively long history: the first clinical trials were initiated several decades ago. Since then, nanocarriers composed of synthetic polymers, liposomes, and dendrimers have been investigated, and a few have found clinical applications (see Peer *et al.*[1] for a recent review). Considerably less effort has been invested in the development of vehicles based on virus-like particles (VLPs), even though they possess several features that make them attractive as potential nanocarriers. Here we describe a nanocarrier based on RNA bacteriophage MS2, the VLPs of which self-assemble from 180 copies of a single coat protein (13.7 kDa) into a monodisperse, 27.5-nm icosahedral capsid. The periodicity of the capsid, the presence of surface-accessible amino acids with reactive moieties (*e.g.* lysine and glutamic acid residues), and the tolerance of a single-chain version of the coat protein dimer to diverse peptide insertions[2] enable dense, repetitive display of targeting peptides either by chemical conjugation or genetic insertion, and display of aptamers, vitamins, glycoproteins, *etc.* by chemical conjugation.[3–9] MS2 VLPs, furthermore, possess a relatively large interior volume that can be loaded with a variety of materials using several approaches.[4,6,8,9] In particular, the ability of MS2 coat protein to spontaneously assemble in the presence of nucleic acids allows the particle to be loaded with therapeutic RNAs or with RNA-conjugated drugs and imaging agents. *In vitro* assembly of VLPs from isolated subunits is most effectively stimulated by a 19-nucleotide RNA stem-loop that specifically interacts with coat protein and normally mediates encapsidation of the viral genome and translational repression of viral replicase synthesis.[7,10,11] Conjugation of this so-called *pac* site to a non-nucleic acid molecule (*e.g.* a protein) causes the molecule to be packaged within the capsid.[7,8] Coat protein also efficiently encapsidates other types of RNA, making MS2 VLPs readily adaptable to packaging RNAs with therapeutic potential (*e.g.* siRNA).[11] MS2 VLPs are, additionally, biocompatible, biodegradable, stable under a variety of temperature, pH, and solvent conditions, and easily synthesized and purified in relatively large quantities.[12] Importantly, Peabody, *et al.* recently reported the use of MS2 VLPs as a platform for random peptide display and affinity selection,[2,13] raising the possibility that a single particle can be used both for identification of cell-targeting peptides and for specific delivery of cargo.

Here we report the delivery of several chemically diverse therapeutic and imaging agents to human hepatocellular carcinoma (HCC) using MS2 VLPs modified with high densities of a targeting peptide (SP94) that binds to HCC. The SP94 peptide was previously identified by affinity selection from a phage display library using HCC targets.[14] The possibility of its chemical conjugation to MS2 VLPs provided a convenient means to test the general suitability of the particles for cell-specific delivery. We loaded MS2 VLPs with a variety of cargo molecules using an *in vitro* assembly reaction, modified the resulting particles with SP94, and tested their ability to deliver the various cargo molecules to HCC in culture.

Results

RNA-Driven Assembly of MS2 Coat Protein Enables Encapsidation of Therapeutic and Imaging Agents within VLPs

The techniques we used to encapsidate therapeutic molecules (chemotherapy drugs, siRNA, and ricin toxin A-chain) and an imaging agent (water-soluble CdSe/ZnS quantum dots) within MS2 VLPs are detailed in the Methods section. To summarize, we first conjugated quantum dots, drugs, and ricin toxin A-chain to *pac* site RNA using an appropriate crosslinker. Molar ratios of cargo molecules to *pac* site RNA were determined to be: 1:80 for Qdot® 585 ITK™ amino(PEG) quantum dots, 0.9:1 for doxorubicin (DOX), 1.1:1 for cisplatin, 3:1 for 5-fluorouracil (5-FU), and 1:1 for ricin toxin A-chain (RTA). We then added cargo-*pac* site conjugates to dimerized coat protein, obtained *via* disassembly of MS2 (or Qβ) virions. Buffered, RNA-modified cargos, as well as siRNA in the absence of the *pac* site, drive *in vitro* assembly of VLPs with cargo encapsulated in the interior volume of the 27.5-nm capsid. After removal of excess coat protein and unencapsidated cargos, the exterior VLP surface was modified with an HCC-specific peptide (SP94, H_2N-SFSIIHTPILPL-COOH[14]), a fusogenic peptide (H5WYG, H_2N-GLFHAIAHFIHGGWHGLIHGWYG-COOH[15]), and PEG-1000. Electron microscopy demonstrates that the resulting particles have morphologies indistinguishable from that of wild-type phage; see Figure 1 for a representative electron micrograph of siRNA-loaded VLPs. Furthermore, the relative concentrations of cargo molecules and coat protein in populations of VLPs loaded with drugs, siRNA, or RTA indicate that nearly 100% of fully-assembled VLPs contain encapsidated cargo. VLPs that encapsidate CdSe/ZnS quantum dots are the exception: each VLP encapsidates an average of one quantum dot, but ~40% of VLPs in the population are ‘empty’. See Figure 1 for a schematic depiction of the above process.

SP94-Targeted, Cargo-Loaded VLPs are Selectively Endocytosed by HCC

To determine whether SP94-targeted VLPs are capable of selectively delivering to HCC the therapeutic and imaging agents described above, we prepared a cocktail of particles, each individually loaded with: (i) ricin toxin A-chain modified with an Alexa Fluor® 488-labeled *pac* site, (ii) *pac* site-modified Qdot® 585 ITK™ amino(PEG) quantum dots, (iii) *pac* site-modified doxorubicin, which is naturally fluorescent, or (iv) Alexa Fluor® 647-labeled siRNA. Assembled VLPs were extensively purified using size-exclusion chromatography, chemically conjugated with SP94 at an approximate density of 60 peptides per VLP, fluorescently-labeled with Alexa Fluor® 532 (Figure 2A) or Alexa Fluor® 555 (Figures 2B, 2C, 3, and 4), and incubated with the HCC cell line, Hep3B, for 15–60 minutes at 37°C. Since each of the four cargos, as well as the Alexa Fluor® 532-labeled capsid, possessed unique spectral characteristics, we were able to individually track them using hyperspectral confocal fluorescence microscopy. Figure 2A indicates that SP94-targeted VLPs can efficiently deliver drugs, siRNA, protein toxins, and quantum dots to HCC, while the representative confocal fluorescence microscopy images shown in Figures 2B and 2C demonstrate that SP94-targeted VLPs are rapidly internalized by HCC ($t_{½}$ = 6 minutes, as

determined by time-course experiments) but show minimal surface binding and no internalization when exposed to normal hepatocytes. We employed flow cytometry to quantify the average number of SP94-targeted VLPs that are internalized by 1×10^6 cells within 1 hour at 37°C and found that each Hep3B cell internalizes an average of 1459 ± 89 VLPs, while each hepatocyte internalizes an average of only 28 ± 19 VLPs; the VLP capsid was labeled with pHrodo™ (a pH-sensitive red fluorophore, the emission of which increases dramatically at pH ≤ 6.0) in these experiments to ensure that surface-bound VLPs were excluded. We also quantified the degree to which various surface modifications alter internalization efficacy and found negligible uptake of the following VLPs by Hep3B and hepatocytes: (1) VLPs modified with ~60 copies of an irrelevant peptide (see discussion of the 'control' peptide in the next section); (2) VLPs modified with ~75 copies of a histidine-rich fusogenic peptide (see discussion of the 'H5WYG' peptide below); (3) VLPs modified with ~145 molecules of PEG-1000; and (4) unmodified VLPs. We, furthermore, observed little difference between the uptake of VLPs that were surface-modified with Alexa Fluor® 555 and VLPs that encapsidated Alexa Fluor 555®-labeled tRNA, which indicates that the fluorescent molecules we used to track SP94-targeted VLPs do not themselves promote internalization (data not shown).

As is evident in Figures 2A and 2B, SP94-targeted VLPs have a punctate appearance within HCC, which indicates that internalization occurs *via* an endocytotic pathway. Further evidence is provided by the fact that SP94-targeted VLPs are largely co-localized with markers for early (Rab5) and late (Rab7) endosomes (data not shown). Fluorescence co-localization experiments demonstrate that SP94-targeted VLPs are directed to lysosomes upon endocytosis by HCC, as evidenced by the positive Pearson's correlation coefficient (r) between SP94-targeted VLPs and lysosomal-associated membrane protein 1 (LAMP-1), as well as the near-zero r-value between SP94-targeted VLPs and Rab11a, a marker for recycling endosomes (Figure 3). Since some therapeutic molecules (*e.g.* siRNA and protein toxins) are expected to be susceptible to lysosomal degradation, we anticipated the need to promote endosomal escape by further modifying VLPs with a histidine-rich fusogenic peptide (H5WYG) that becomes protonated upon acidification ($pK_a = 6.0$) and induces osmotic swelling and endosomal membrane destabilization. As demonstrated by Figure 4, VLPs that co-display the SP94 and H5WYG peptides become dispersed in the cytosol of HCC cells within 1 hour of endocytosis, while VLPs that display the SP94 peptide alone remain in endosomes. As demonstrated below, cytosolic dispersion of VLPs and their encapsidated cargos is critical to retain the activity of siRNA and ricin toxin A-chain.

Multivalent Display of Targeting Peptides Enhances the Avidity of MS2 VLPs for HCC

Multivalent display of targeting ligands is known to promote high avidity interactions between nanoparticles and target cells.[6,16–18] To determine the minimum peptide density necessary to promote maximum avidity for HCC, we conjugated various densities of SP94 to the surface of MS2 VLPs using an extended-length, heterobifunctional crosslinker, $SM(PEG)_{24}$; under the conditions described in the Methods section, each VLP can be modified with a maximum of 246 ± 9 (mean ± s.d.) peptides, and peptide density can be reduced in a precise manner by altering the reaction stoichiometry, time, and/or temperature. We then fluorescently-labeled SP94-targeted VLPs and employed flow cytometry to quantify their avidity for various HCC cell lines (Hep3B, PLC/PRF/5, and HepG2), as well as non-transformed hepatocytes and other control cells (endothelial cells, mononuclear cells, and lymphocytes). Figure 5A shows typical saturation binding curves, which demonstrate that VLPs bearing ~60 copies of the SP94 peptide bind to Hep3B in a concentration-dependent fashion but show minimal binding to normal hepatocytes, even at high VLP concentrations. In order to directly compare the avidities of targeted and non-targeted VLPs for various cell lines, we used saturation binding curves to calculate apparent dissociation

constants (K_d values), which are a measure of specific surface binding and are inversely related to avidity (*i.e.* low dissociation constants are indicative of high affinity or avidity). As demonstrated by Figure 5B, the avidity of MS2 VLPs for HCC is strongly dependent on the density of targeting peptides displayed on the capsid surface, as well as (to a lesser extent) the inherent affinity of the targeting peptide for HCC. K_d values of SP94-targeted VLPs for Hep3B remain essentially constant when they display between ~60 and ~240 peptides/particle. As density decreases to ~30 peptides/particle, however, the avidity of SP94-targeted VLPs for Hep3B starts to decline (*i.e.* K_d values start to increase). K_d values of MS2 VLPs modified with a second HCC-specific peptide (SP88, H_2N-ELMNPLLPFIQPGGC-COOH[14]) also decrease with increasing peptide density, but higher densities are necessary to achieve comparable avidity, presumably due to the peptide's lower intrinsic affinity for Hep3B (K_d = 489 ± 31.4 nM for monovalent SP88 vs. 211 ± 12.2 nM for monovalent SP94). Importantly, control experiments demonstrate that SP94-targeted VLPs bind selectively to several HCC cell lines over hepatocytes and other control cells. As shown in Figure 5C, MS2 VLPs decorated with ~60 copies of SP94 have a 10^4-fold higher avidity for Hep3B, PLC/PRF/5, and HepG2 than for human hepatocytes, endothelial cells (HUVECs), and immune cells (peripheral blood mononuclear cells (PBMCs), B-lymphocytes, and T-lymphocytes). Furthermore, SP94-targeted VLPs have a 6000-fold higher avidity for Hep3B than VLPs bearing ~60 copies of an irrelevant peptide ('Control Peptide', H_2N-FPWFPLPSPYGNGGC-COOH[14]) and a 45,000-fold higher avidity for Hep3B than unmodified VLPs, both of which demonstrate that the SP94 peptide promotes specific binding to HCC.

It is important to note that the VLPs used for targeted delivery of therapeutic molecules were routinely PEGylated in the expectation that, when they are eventually tested *in vivo*, this should minimize proteolytic degradation, reduce the anti-VLP humoral immune response, and mitigate non-specific interactions with non-target cells, all of which should increase the circulation half-life and enhance bioavailability of encapsidated cargo(s).[5] Coupling methyl-$(PEG)_{24}$-amine (MW ≈ 1000 Da; length ≈ 8.6 nm) to the MS2 capsid using EDC results in > 80% modification and reduces recognition of MS2 VLPs by anti-MS2 polyclonal antibodies (data not shown). Masking a nanoparticle's surface with PEG, however, can reduce its affinity for the target cell by interfering with ligand binding.[19] Therefore, we tested the degree to which PEGylated VLPs, surface-modified with SP94 using $SM(PEG)_{24}$ (spacer arm ≈ 9.5 nm), bind to Hep3B and hepatocytes and found that PEG-1000 does not substantially affect the specific avidity of SP94-targeted VLPs for HCC but does result in a 2-fold reduction in the non-specific binding of SP94-targeted VLPs to hepatocytes (see Figure 5D). For these reasons, VLPs used in the targeted delivery experiments described below were routinely PEGylated as detailed in the Methods section.

Delivery of a Chemotherapeutic Drug Cocktail to HCC *via* SP94-Targeted VLPs Induces Selective Cytotoxicity

Having established that the SP94 peptide promotes selective binding and internalization of VLPs by HCC, we then tested the ability of SP94-targeted VLPs to effectively deliver the chemotherapeutic drugs, doxorubicin (DOX), cisplatin, and 5-fluorouracil (5-FU), a cocktail known to be particularly effective against HCC.[20] Hep3B cells express moderate levels of P-glycoprotein (Pgp), an efflux pump that is typically upregulated by cells during acquisition of a $MDR1^+$ phenotype and that results in reduced intracellular accumulation of certain drugs, including anthracyclines like DOX.[21] To enhance the natural resistance of Hep3B to anthracyclines, we incubated parental cells in increasing concentrations of DOX for a period of three weeks. The concentration of free DOX necessary to kill 50% of Hep3B with an induced $MDR1^+$ phenotype (LC_{50}) is 285.6 ± 8.2 nM, a value that can be reduced *via* inhibition of Pgp using cyclosporin A (CsA)[21] or by addition of cisplatin and 5-FU, neither

of which are substrates for Pgp[20,22] (see Figure 6A). Targeted nanocarriers internalized *via* receptor-mediated endocytosis are typically able to circumvent Pgp efflux mechanisms and can, therefore, kill MDR cancer cells at lower drug concentrations.[23,24] To this end, we loaded MS2 and Qβ VLPs with an estimated 79 ± 6 molecules of DOX (per VLP). Figure 6A shows that DOX-loaded, SP94-targeted VLPs kill $MDR1^+$ Hep3B at IC_{50} values of 10–15 nM, a 20-fold improvement in the cytotoxicity of free DOX. Moreover, VLPs loaded with DOX (38 ± 4 per VLP), cisplatin (43 ± 5 per VLP), and 5-FU (111 ± 13 per VLP) kill 50% of $MDR1^+$ Hep3B at concentrations < 1 nM (Figure 6A). Figure 6B compares the time-dependent (24 hours and 7 days) cytotoxicity of DOX and the DOX/cisplatin/5-FU cocktail, when delivered to $MDR1^+$ Hep3B and normal hepatocytes in their free forms or encapsidated within SP94-targeted MS2 and Qβ VLPs. Free drugs are highly toxic to both Hep3B and hepatocytes, as evidenced by the near-zero *via*bility of cells exposed to the cocktail for 24 hours. In contrast, encapsidation of the drug(s) within SP94-targeted VLPs confers a high degree of specificity for HCC: nearly 100% of $MDR1^+$ Hep3B cells are dead within 7 days, while hepatocyte viability remains relatively unaffected. We attribute the 10% decrease in hepatocyte viability induced by DOX-loaded MS2 VLPs to time-dependent degradation of the capsid, which, over a period of 7 days, releases a sufficient concentration of free DOX to kill some hepatocytes. When we employed SP94-targeted VLPs of Qβ, a 28.5-nm icosahedral bacteriophage that is more stable than MS2 due to disulfide crosslinking of its capsid, $MDR1^+$ Hep3B cells were killed with similar efficacy, but long-term cytotoxicity of hepatocytes was largely mitigated (Figure 6B).

SP94-Targeted VLPs Loaded With a Cocktail of Anti-Cyclin siRNAs Induce Growth Arrest and Apoptosis of HCC

The activation of certain cyclins and cyclin-dependent kinases (Cdks), including cyclin A, cyclin D1, cyclin E, and Cdk4, has been implicated in hepatocarcinogenesis.[25] Furthermore, siRNA-mediated silencing of cyclin B1 and cyclin E enhances the susceptibility of various cancer types to chemotherapeutic drugs and induces growth arrest and apoptosis of HCC.[26–28] To test the suitability of MS2 VLPs for siRNA delivery, we loaded them with a cocktail of siRNAs that silence expression of cyclin A2, cyclin B1, cyclin D1, and cyclin E1. The siRNA mixture induced assembly of intact capsids in the absence of the *pac* site (see the TEM image in Figure 1), resulting in VLPs that encapsidate an average of 84 ± 3 RNA molecules (per VLP). We, additionally, found that siRNA-loaded VLPs are stable for > 3 months when stored in 1X PBS at 4°C and protect encapsidated siRNA from RNase-mediated degradation (data not shown). SP94-targeted VLPs that encapsidate the siRNA cocktail induce apoptosis in > 90% of Hep3B within 36 hours at a siRNA concentration of 150 pM without substantially affecting the viability of hepatocytes (see Figure 7A). To determine the mechanism by which cyclin-specific siRNAs induce apoptosis of Hep3B, we also assayed for proliferation, growth arrest, and cyclin protein concentrations. As demonstrated by Figure 7B, SP94-targeted VLPs loaded with cyclin A2, D1, and E1-specific siRNAs reduce the number of proliferating cells from ~80% to ~10% and increase the number of cells in G_0/G_1 from ~25% to ~90% within 72 hours at a siRNA concentration of 150 pM; the cyclin B1-specific siRNA was omitted from this experiment since it induced G_2/M arrest and, therefore, made it difficult to discern a trend in the percentage of G_0/G_1-arrested cells versus time. SP94-targeted VLPs loaded with cyclin-specific siRNAs also induce a dose-dependent (Figure 7C) and time-dependent (Figure 7D) decrease in the concentrations of target proteins, presumably due to siRNA-mediated degradation of cyclin mRNAs; to confirm this hypothesis, we used real-time PCR to demonstrate that SP94-targeted VLPs loaded with the cyclin A2-specific siRNA induce a dose- and time-dependent decrease in cyclin A2 mRNA concentrations (see the red curves in Figures 7C and 7D). As determined from the dose-response curves shown in Figure 7C, the concentrations of siRNA necessary to silence 90% of cyclin A2, B1, D1, and E1 protein expression (IC_{90}) within 48

hours when delivered to Hep3B *via* SP94-targeted VLPs are 152 ± 4.3 pM, 164 ± 2.5 pM, 171 ± 3.9 pM, and 201 ± 7.8 pM, respectively. These IC_{90} values are all within 10% of the silencing efficacy achieved by delivering the same siRNAs to Hep3B using the commercially-available transfection reagent, Lipofectamine™ RNAiMAX (see Figure 8A). Unlike the non-specific transfection reagent, however, SP94-targeted VLPs are able to selectively silence cyclin expression in Hep3B without affecting cyclin concentrations in hepatocytes (see Figure 8B). It should be noted that the VLPs used to deliver siRNA cocktails were always co-modified with SP94 and H5WYG; SP94-targeted, siRNA-loaded VLPs induce apoptosis in fewer than 15% of Hep3B cells in the absence of the H5WYG fusogenic peptide.

Ricin Toxin A-Chain Induces Specific Intoxication of HCC at Femtomolar Concentrations when Delivered by SP94-Targeted VLPs

Ricin is a potent protein toxin derived from the seeds of *Ricinis communis* and exerts its cytotoxic effect *via* catalytic inhibition of protein synthesis. Native ricin is a heterodimer composed of the catalytically-active A-chain, which inactivates eukaryotic ribosomes, and the galactose-binding B-chain, which utilizes its lectin activity to promote receptor-mediated endocytosis of the toxin.[29,30] Brown *et al*. have demonstrated that MS2 VLPs can be loaded with ricin toxin A-chain (RTA), modified with transferrin (Tf), and delivered to a human leukemic cell line, HL-60, through interaction with the transferrin receptor.[7] In these experiments, Tf-targeted, RTA-loaded VLPs caused a > 90% reduction in HL-60 viability at a concentration of ~10 nM; in comparison, non-targeted, RTA-loaded VLPs and RTA alone killed ~60% and ~40% of cells, respectively, at the same reagent concentration. We employed a similar technique to encapsidate RTA in SP94-targeted MS2 VLPs (34 ± 8 RTA molecules per VLP, on average) and observed a dose-dependent (Figure 9A) and time-dependent (Figure 9B) induction of apoptosis in HCC, as determined by activation of caspase-3. At an RTA concentration of 100 fM, ~90% of Hep3B cells become positive for caspase-3 activation within 72 hours, while hepatocyte viability is essentially unaffected by a 10^4-fold higher RTA concentration (see Figure 9A), even after continual exposure for 2 weeks (data not shown). Induction of apoptosis correlates with a dose- and time-dependent decrease in nascent protein synthesis, as demonstrated by Figures 9C and 9D, respectively. The concentration of RTA that is necessary to reduce protein biosynthesis by 90% (IC_{90}) when delivered to Hep3B *via* SP94-targeted VLPs is 98.9 ± 3.8 fM. The IC_{90} value of RTA-loaded, SP94-targeted VLPs causes a 90% reduction in nascent protein synthesis when exposed to Hep3B for 48 hours but has little effect on hepatocytes (see Figure 10A). We observe that neither non-targeted, RTA-loaded VLPs nor monomeric RTA has an effect on protein synthesis when exposed to Hep3B and hepatocytes, presumably due to the lack of cell-specific interactions needed to induce internalization (Figure 10A). Conversely, RTA-loaded MS2 VLPs, when modified with an octaarginine peptide that promotes non-specific macropinocytosis,[31] induce rapid inhibition of protein synthesis in both Hep3B and hepatocytes (Figure 10A). It is important to note that the ability of SP94-targeted, RTA-loaded VLPs to effectively induce apoptosis of HCC depends on the presence of the H5WYG fusogenic peptide, likely because it promotes endosomal escape and prevents lysosomal degradation of RTA (see Figure 10B). Further evidence that endosomal escape of RTA-loaded VLPs is critical to induce cytotoxicity of target cells is provided by the fact that chloroquine, an agent that inhibits lysosome acidification, restores the ability of RTA-loaded VLPs modified with the SP94 peptide alone to induce activation of caspase-3 in Hep3B (Figure 10B). Using our most effective design (*i.e.* RTA-loaded VLPs modified with ~60 SP94 peptides, ~75 H5WYG peptides, and ~145 PEG-1000 molecules), we have found that an average VLP: cell ratio of approximately 2.5 is sufficient to kill nearly the entire population of Hep3B cells.

Discussion

The normal function of a virus particle is to deliver molecular cargo (normally the viral genome) to a target cell, but, thus far, comparatively little effort has been invested in repurposing VLPs for delivery of drugs and imaging agents.[32,33] The majority of such studies have focused on particles derived from Cow Pea Mosaic Virus (CPMV) or Canine Parvo Virus (CPV). CPMV has the ability to bind to and become internalized by a wide variety of mammalian cells since it naturally interacts with the cell-surface protein, vimentin.[34] In attempts to confer cell-type specificity, several other targeting ligands (*e.g.* transferrin and folic acid) have been conjugated to the particle, with some success at directing it selectively to tumor cells. Although cell-specific binding has been demonstrated, we are unaware of attempts to utilize CPMV-based particles for drug delivery. Fortuitously, CPV naturally uses the transferrin receptor for cell entry during infection, and the over-expression of transferrin receptor on many tumor cell types makes the particle an attractive candidate for delivery applications. Although fluorescently-labeled particles show selective binding and entry into tumor cells, CPV has not yet been utilized specifically for drug delivery.[35]

A few previous efforts to use MS2 VLPs for both drug delivery and imaging purposes have been reported, and the results have been encouraging. Wu *et al*., for example, reported the delivery of ricin toxin A-chain (RTA) to leukemic HL-60 cells using VLPs decorated with intact transferrin protein,[7] thus taking advantage of transferrin receptor over-expression to selectively target tumor cells. These particles killed ~75% of HL-60 cells at a RTA concentration of ~1 nM, an approximate ten-fold improvement over RTA alone, which, for some reason, exhibited an unexpected level of toxicity. The same group showed that antisense DNA oligonucleotides designed to interfere with p120 expression (an essential nucleolar protein over-expressed in tumors) can be delivered to HL60 cells, again in a transferrin-dependent fashion.[8] However, the required DNA concentration was relatively high ($LC_{50} \approx 100$ nM) and only about 100-fold lower than the naked oligonucleotide added directly to the culture medium.

Our results represent a significant improvement. For example, when delivered by our particles, ricin toxin A-chain causes 90% of Hep3B cells to apoptose within 72 hours at a RTA concentration of only ~100 fM. Moreover, the specificity for HCC cells is remarkable; normal hepatocytes (and a variety of other cell types) are not detectably affected at these concentrations. Our siRNA delivery results are similarly dramatic. SP94-targeted particles that deliver anti-cyclin siRNAs at a concentration of 150 pM induce apoptosis in virtually 100% of Hep3B cells within 48 hours without affecting the viability of normal hepatocytes. We attribute the relative efficiency of our VLPs to several factors: (i) Multivalency of the targeting peptide. Our particles can display up to 240 targeting peptides per VLP, but as few as 60 peptides are sufficient to maximize avidity and promote cell-specific internalization. The importance of multivalency has been previously demonstrated using dendrimers targeted to the folate receptor; in these studies, multivalent presentation of folic acid dramatically enhanced targeting efficacy.[36] (ii) Presence of a fusogenic peptide. We find that the potency of particles containing a protein or nucleic acid cargo (*i.e.* ricin toxin A-chain or siRNA) is greatly enhanced by the presence of a fusogenic peptide, presumably because it promotes endosomal escape of these molecules before they can be degraded in lysosomes. (iii) PEGylation. Nanocarriers of diverse types benefit from a coating of PEG.[5,19] Our particles are more stable and show less non-specific binding activity when PEGylated. (iv) Receptor identity and ligand specificity. Although the receptor recognized by SP94 has not yet been identified, it is apparently present in high densities on HCC and mediates efficient entry by endocytosis. Moreover, it seems to be present specifically on HCC, and its absence from the other cell types we tested surely contributes to the high

specificity we observe. (v). Choice of drug. Ricin toxin A-chain and anti-cyclin siRNAs are potent inducers of apoptosis. When combined with an efficient and highly specific delivery vehicle, they selectively kill target cells at sub-nanomolar concentrations.

Conclusions

MS2 VLPs can be readily adapted for specific delivery of a variety of molecular cargos to diverse cell types. Due to their natural ability to encapsidate nucleic acids, they are especially well-suited for delivering RNA- and DNA-based drugs, but they can also encapsulate diverse, non-nucleic acid cargos (*e.g.* quantum dots, chemotherapy drugs, and protein toxins) when the cargo molecules are linked to the MS2 *pac* site. In the experiments described here, we decorated MS2 VLPs with SP94, an HCC-specific peptide, but other targeting strategies are possible. Indeed, prior work reported the use of transferring,[7,8] of a DNA aptamer that binds to a tyrosine kinase receptor,[6] and of folic acid for this purpose.[37] The MS2 VLP platform has a special advantage when peptides are used for targeting, however. We previously showed that peptides can be displayed on the VLP surface by genetic fusion to coat protein and that these VLPs encapsidate the mRNA that encodes the fusion protein.[2] On this basis, we created a system for VLP-based peptide display analogous to conventional filamentous phage display, which allows for affinity selection of arbitrary binding activities from complex random sequence peptide libraries.[13] Selected sequences are then recovered by reverse transcription and polymerase chain reaction and re-cloned for synthesis of the selected VLPs in bacteria. The existence of this affinity selection capability means that a single particle can serve both for identification of cell-specific targeting ligands and as a drug delivery vehicle.

Materials and Methods

Materials

Antibodies against LAMP-1 (rabbit pAb), Rab11a (mouse mAb), cyclin A2 (mouse mAb), cyclin B1 (mouse mAb), cyclin D1 (mouse mAb), and cyclin E1 (mouse mAb) were purchased from Abcam, Inc. (Cambridge, MA). *Silencer* select siRNAs (siRNA IDs for cyclin A2, B1, D1, and E1 are s2513, s2515, s229, and s2526, respectively) and the TaqMan® Fast Cells-to-CT™ Kit were purchased from Applied Biosystems™ by Life Technologies Corporation (Carlsbad, CA). Human Hep3B (HB-8064), human hepatocytes (CRL-11233), human peripheral blood mononuclear cells (CRL-9855), human umbilical cord vein endothelial cells (CRL-2873), T lymphocytes (CRL-8293), B lymphocytes (CCL-156), Eagle's Minimum Essential Medium (EMEM), Dulbecco's Modified Eagle's Medium (DMEM), Iscove's Modified Dulbecco's Medium (IMDM), RPMI 1640 medium, fetal bovine serum (FBS), and 1X trypsin-EDTA solution (0.25% trypsin with 0.53 mM EDTA) were purchased from American Type Culture Collection (ATCC; Manassas, Virginia). CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (485/535) was purchased from BioVision, Inc. (Mountain View, CA). Reactive forms of the *pac* site were synthesized by Integrated DNA Technologies (Coralville, IA). Hoechst 33342 (350/461), 4′, 6-diamidino-2-phenylindole (DAPI, 356/451), CellTracker™ Violet BMQC (415/516), CellTracker™ Green CMFDA (492/517), Ulysis™ Alexa Fluor® 488 Nucleic Acid Labeling Kit (495/519), Alexa Fluor® 488 Antibody Labeling Kit (495/519), Alexa Fluor® 488 conjugate of annexin V (495/519), Alexa Fluor® 488-labeled mouse monoclonal antibody to BrdU (clone MoBU-1) (494/519), Alexa Fluor® 488 goat anti-mouse IgG (H+L) (495/519), Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (495/519), Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay (495/519), SYTOX® Green nucleic acid stain (504/523), Alexa Fluor® 532 carboxylic acid succinimidyl ester (532/554), Alexa Fluor® 555 C_2 maleimide (555/565), Alexa Fluor® 555 hydrazide (555/565), Alexa Fluor® 555 carboxylic acid succinimidyl ester (555/565), Qdot® 585 ITK™ amino(PEG) (300/585), pHrodo™

succinimidyl ester (560/586), propidium iodide (535/617), Ulysis™ Alexa Fluor® 647 Nucleic Acid Labeling Kit (650/668), Alexa Fluor® 647 conjugate of annexin V (650/668), Alexa Fluor® 647 goat anti-mouse IgG (H+L) (650/668), *SlowFade*® Gold antifade reagent (with and without DAPI), Image-iT® FX signal enhancer, Lipofectamine™ RNAiMAX, 1X Dulbecco's phosphate-buffered saline (D-PBS), bovine albumin fraction V solution (BSA, 7.5%), and transferrin were purchased from Invitrogen Life Sciences (Carlsbad, CA). BEGM Bullet Kits were purchased from Lonza Group Limited (Clonetics; Walkersville, MD). Amicon® Ultra-4 Centrifugal Filter Units (3 kDa, 50 kDa, and 100 kDa MWCO) were purchased from Millipore (Billerica, MA). All peptides were synthesized by New England Peptide (Gardner, MA). Sulfosuccinimidyl 6-(3′-[2-pyridyldithio]-propionamido)hexanoate (Sulfo-LC-SPDP), succinimidyl-[(*N*-maleimidopropionamido)-tetracosaethyleneglycol] ester (SM(PEG)$_{24}$), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), methyl-(ethyleneglycol)$_{24}$amine (MA(PEG)$_{24}$), Slide-A-Lyzer MINI Dialysis Units (20 kDa MWCO), and Slide-A-Lyzer G2 Dialysis Cassettes (3.5 kDa MWCO) were purchased from Pierce Protein Research Products (Thermo Fisher Scientific LSR; Rockford, IL). Ultra pure, EM-grade formaldehyde (16%, methanol-free) was purchased from Polysciences, Inc. (Warrington, PA). Sephadex™ G-75, Triton® X-100 hexadecane (≥ 99%), cyclosporin A from *Tolypocladium inflatum* (CsA, > 95%), 5-bromo-2′-deoxyuridine (BrdU, ≥ 99%), chloroquine diphosphate salt (≥ 98%), human epidermal growth factor, L-α-phosphatidylethanolamine, thymidine (≥ 99%), hypoxanthine (≥ 99%), bovine fibronectin, bovine collagen type I, gelatin, soybean trypsin inhibitor (≥ 98%), DMEM without phenol red, goat serum, doxorubicin hydrochloride (≥ 98%), 5-fluorouracil (≥ 99%), *cis*-diammineplatinum(II) dichloride (cisplatin, ≥ 99.9%), and deglycosylated A-chain from *Ricinus communis* were purchased from Sigma-Aldrich (St. Louis, MO). Spectra/Por® dialysis tubing (15 kDa MWCO) was purchased from Spectrum® Laboratories, Inc. (Rancho Dominguez, CA).

Cell Culture

Hep3B, HepG2, PLC/PRF/5, hepatocytes, PBMCs, T-lymphocytes, and B-lymphocytes were obtained from ATCC and grown per manufacturer's instructions. Briefly, Hep3B, HepG2, and PLC/PRF/5 were maintained in EMEM with 10% FBS. Hepatocytes were grown in flasks coated with BSA, fibronectin, and bovine collagen type I; the culture medium used was BEGM (gentamycin, amphotericin, and epinephrine were discarded from the BEGM Bullet kit) with 5 ng/mL epidermal growth factor, 70 ng/mL phosphatidylethanolamine, and 10% FBS. HUVECs were grown in DMEM with 20% FBS; gelatin-coated flasks were used to promote adhesion. PBMCs, T lymphocytes, and B lymphocytes were maintained in suspension flasks (Greiner Bio-One; Monroe, NC). PBMCs were grown in IMDM supplemented with 0.02 mM thymidine, 0.1 mM hypoxanthine, 0.05 mM 2-mercaptoethanol, and 10% FBS. T and B lymphocytes were grown in IMDM with 20% FBS and RPMI 1640 medium with 20% FBS, respectively. All cells were maintained at 37°C in a humidified atmosphere (air supplemented with 5% CO_2). Adherent cells were passaged with 0.05% trypsin at a sub-cultivation ratio of 1:3, while non-adherent cells were seeded at a density of ~1×10^5 cells/mL and maintained at 1–5×10^6 cells/mL.

Disassembly of MS2 and Qβ Bacteriophages

MS2 and Qβ bacteriophages were produced by infecting *E. coli* A/λ using well-established procedures[38] and purified by sedimentation to equilibrium in CsCl density gradients. To harvest coat protein dimers for reassembly reactions, MS2 phage (10 mg/mL) was diluted 1:3 in cold glacial acetic acid, incubated for two hours at 4°C, and centrifuged briefly to pellet precipitated RNA and maturase. Qβ phage (10 mg/mL) was incubated in 50 mM Trizma® hydrochloride with 6 M urea and 10 mM dithiothreitol (pH 8.5) for 1 hour at 4°C and centrifuged briefly to remove precipitated RNA. The resulting protein was dialyzed

against 10 mM acetic acid with 50 mM NaCl (~pH 4) using regenerated cellulose tubing (15 kDa MWCO); buffer (1.5 L) was changed twice in 12-hour intervals. Dialyzed protein was then applied to a 0.9 × 45 cm Sephadex™ G-75 column and eluted using 10 mM acetic acid with 50 mM NaCl (~pH 4). ~1 mL fractions were collected and assayed for protein content *via* agarose gel electrophoresis; gels (1%) were stained with Coomassie® Brilliant Blue R-250 to enable visualization of protein. Fractions that contained coat protein dimers (~28 kDa) were pooled, concentrated using a centrifugal filter unit (10 kDa MWCO), resuspended in 10 mM acetic acid with 50 mM NaCl (~pH 4), and stored at 4°C for no more than two weeks. The concentrations of coat protein dimers were measured using UV spectroscopy immediately before their use in re-assembly reactions.

Modification of Various Therapeutic and Diagnostic Agents with the *pac* Site

Various reactive forms of the *pac* site were synthesized, including those with: (1) a 3′ sulfhydryl moiety, (2) a 3′ amine moiety, or (3) three internal 5-fluorouridines and a 5′ thiol group. All *pac* site derivatives contained a uracil spacer (3–9 nucleotides in length) between the wild-type sequence and the reactive group. To prepare quantum dot-*pac* site conjugates, Qdot® 585 ITK™ amino(PEG), a CdSe/ZnS (core/shell) quantum dot passivated with amine-terminated PEG, was diluted to 1 μM with 1X PBS and incubated with a 10-fold molar excess of Sulfo-LC-SPDP, a thiol-cleavable, amine-to-sulfhydryl crosslinker, for 2 hours at room temperature; excess crosslinker was removed *via* centrifugal filtration (50 kDa MWCO). SPDP-activated quantum dots were then incubated with a 10-fold molar excess of the sulfhydryl-modified *pac* site for 12 hours at 4°C. Unreacted RNA was removed using a centrifugal filter device (50 kDa MWCO), and quantum dot-*pac* site conjugates were resuspended in 50 mM Tris-HCl (pH 8.5) at a quantum dot concentration of 10 μM. The average number of *pac* sites per quantum dot was determined by exposing 5 μL of the conjugate to 10 mM DTT for 1 hour at room temperature, dialyzing the resulting solution to remove liberated *pac* site molecules (Slide-A-Lyzer MINI Dialysis Units, 20 kDa MWCO), and measuring the absorbance of the solution at 260-nm (A_{260}) before and after RNA removal.

To prepare doxorubicin-*pac* site conjugates, the sulfhydryl-modified *pac* site was diluted to 5 μM with 1X PBS and incubated with a 10-fold molar excess of Sulfo-LC-SPDP and a 5-fold molar excess of doxorubicin (DOX), which contains a primary amine group, for 2 hours at room temperature and then 12 hours at 4°C. The reaction mixture was dialyzed (Slide-A-Lyzer Cassette, 3.5 kDa MWCO) against 50 mM Tris-HCl (pH 8.5) to remove unreacted crosslinker and drug; conjugates were concentrated *via* centrifugal filtration (3 kDa MWCO) and resuspended in 50 mM Tris-HCl (pH 8.5) at a RNA concentration of 50 μM. The degree of labeling (*i.e.* the number of DOX molecules per *pac* site) was determined using the following equation:

$$\text{Degree of Labeling} = \frac{A_{484} * \text{molecular weight of } pac \text{ site}}{[\text{pac site (mg/mL)}] * \varepsilon_{DOX}}$$

Where A_{484} is the absorbance of the DOX-pac site conjugate at 484 nm (λ_{max} for DOX) and $\varepsilon_{DOX} = 10{,}800\ cm^{-1}M^{-1}$.

To prepare *pac* sites modified with a combination of DOX, cisplatin, and 5-fluorouracil (5-FU), the *pac* site with three internal 5-fluorouridines and a 5′ thiol group was first reduced *via* exposure to 10 mM DTT for 2 hours at room temperature and desalted using a polyacrylamide desalting column (1.8 kDa MWCO). 5 μM of the reduced *pac* site was incubated for 2 hours at room temperature and then 12 hours at 4°C with a 10-fold molar excess of Sulfo-LC-SPDP and a 5-fold molar excess of DOX and cisplatin, both of which

contain primary amine groups. Unreacted crosslinker and drugs were removed as described above. Conjugates were concentrated *via* centrifugal filtration (3 kDa MWCO) and resuspended in 50 mM Tris-HCl (pH 8.5) at a RNA concentration of 50 μM. The DOX:*pac* site ratio was determined as described above, and the cisplatin:*pac* site ratio was measured according to the technique described by Anilanmert, *et al*.[39].

Ricin toxin A-chain-*pac* site conjugates were synthesized as described previously[7] with minor modifications. Briefly, the amine-modified *pac* site (5 μM in 1X PBS) was incubated with a 10-fold molar excess of Sulfo-LC-SPDP for 2 hours at room temperature and then 12 hours at 4°C; unreacted crosslinker was removed *via* dialysis (Slide-A-Lyzer Cassette, 3.5 kDa MWCO) against 1X PBS. 1 mg of deglycosylated ricin toxin A-chain (RTA) was reduced by exposure to 10 mM DTT for 2 hours at room temperature and desalted using a polyacrylamide desalting column (6 kDa MWCO). Reduced RTA (1 μM in 1X PBS), which contains a free cysteine residue, was immediately combined with a 5-fold molar excess of the SPDP-activated *pac* site, flushed with nitrogen gas, and incubated at room temperature for 4 hours and then at 4°C for 24 hours. Unreacted RNA was removed *via* centrifugal-driven filtration (10 kDa MWCO), conjugates were re-suspended in 50 mM Tris-HCl (pH 8.5) at a RTA concentration of 10 μM, and the crosslinking efficiency was determined using UV spectroscopy and SDS-PAGE. A 10 μg portion of the RTA-*pac* site conjugate was fluorescently-labeled per manufacturer's instructions using the Ulysis™ Alexa Fluor® 488 Nucleic Acid Labeling Kit. It is important to note that Sulfo-LC-SPDP is a thiol-cleavable crosslinker, which enables liberation of cargo from the *pac* site upon exposure to the reducing intracellular environment.

MS2 VLPs that encapsidate cyclin-specific siRNA were synthesized as described below; linkage of siRNA to the *pac* site was unnecessary, however, due to the ability of MS2 coat protein to non-specifically package RNA *in vitro*. 10 μg of cyclin A2-specific siRNA was fluorescently-labeled per manufacturer's instructions using the Ulysis™ Alexa Fluor® 647 Nucleic Acid Labeling Kit.

Encapsidation of Various Cargos within MS2 and Qβ VLPs

Coat protein dimers were concentrated to 1 mM in 10 mM acetic acid with 50 mM NaCl (~pH 4), while siRNA and *pac* site-modified cargos were diluted to 10 μM (total RNA concentration) with 50 mM Tris-HCl (pH 8.5). 1 nmol of dimerized coat protein was then incubated with 1000, 500, 250, 125, or 62.5 pmol of RNA for 1 hour at room temperature, and re-assembly efficiency was assessed using agarose gel electrophoresis as described previously.[40] Based on these results, a four-fold molar excess of dimerized coat protein was used in most re-assembly reactions. For example, 100 μL of *pac* site-modified quantum dots (10 μM of RNA, which corresponds to ~0.125 μM of quantum dots) was combined with 40 μL of MS2 dimers (1 mM). Re-assembly reaction mixtures were applied to a 0.9 × 45 cm Sephadex™ G-75 column in order to remove excess coat protein and unencapsidated cargos. VLPs were eluted using 50 mM Tris-HCl (pH 8.5). ~1 mL fractions were collected and assayed for protein content *via* agarose gel electrophoresis; gels (1%) were stained with Coomassie® Brilliant Blue R-250 to enable visualization of protein. Fractions that contained re-assembled VLPs were pooled and concentrated using a centrifugal filter unit (100 kDa MWCO); VLPs were extensively washed (6–10 times) and re-suspended in 1X PBS.

To determine the average number of cargo molecules per VLP, 10 μL of purified, concentrated VLPs were incubated in 50 mM Tris-HCl (pH 8.5) with 6 M urea and 10 mM DTT for 1 hour at room temperature; the resulting samples were subjected to SDS-PAGE to determine coat protein and RTA concentrations, UV spectroscopy (A_{260}) to determine siRNA and *pac* site concentrations, and fluorimetry to determine quantum dot and DOX concentrations. Re-assembled VLPs were further characterized by electron microscopy

(according to the procedure described by Kovacs, *et al.*[5]) to determine the fraction of intact capsids.

Modification of VLP Capsids with Peptides and PEG

All peptides were synthesized with a C-terminal cysteine residue, separated from the reported sequence by a $(Gly)_2$ spacer, and conjugated to surface lysine residues present in the MS2 (or Qβ) capsid using the heterobifunctional crosslinker, $SM(PEG)_{24}$, which is reactive toward amine and sulfhydryl groups and contains a PEG spacer arm 9.5-nm in length. Crosslinkers with PEG spacers were used to reduce the potential for any steric hindrance of ligand binding and to enable subsequent PEGylation of the MS2 surface, which reduces non-specific interactions and prevents recognition of MS2 VLPs by anti-MS2 antibodies (see below for more details). VLPs were incubated with a 10-fold molar excess of $SM(PEG)_{24}$ for 2 hours at room temperature, and excess crosslinker was removed using a centrifugal filtration device (100 kDa MWCO). Activated capsids were immediately incubated with various concentrations of peptides (0.1 to 10-fold molar excess) for either 1 hour at room temperature or 12 hours at 4°C; unreacted peptides were removed *via* centrifugal filtration. Average peptide density was determined using SDS-PAGE. ImageJ Image Processing and Analysis Software was utilized to compare band intensities relative to a standard concentration curve. The targeting peptide employed in the majority of studies was SP94 (H_2N-SFSIIHTPILPL-COOH[14]), an HCC-specific peptide previously identified by phage display. Some experiments utilized a second targeting peptide (SP88, H_2N-ELMNPLLPFIQP-COOH[14]), which has a slightly lower inherent affinity for HCC than SP94. Control experiments were conducted using VLPs modified with an irrelevant peptide (H_2N-FPWFPLPSPYGN-COOH[14]) that does not bind to HCC. Endosomal escape of internalized VLPs was promoted by a histidine-rich fusogenic peptide (H5WYG, H_2N-GLFHAIAHFIHGGWHGLIHGWYG-COOH[15]), and non-specific macropinocytosis of RTA-loaded VLPs was stimulated using a peptide composed of eight arginine residues ($R8$[31]).

VLPs were PEGylated *via* incubation with a 5-fold molar excess of EDC-activated $MA(PEG)_{24}$ for 12 hours at 4°C; capsids were purified *via* centrifugal filtration (100 kDa MWCO), and the extent of PEGylation was determined using SDS-PAGE, as described previously[5]. The degree to which polyclonal anti-MS2 antibodies bind to PEGylated and non-PEGylated VLPs was determined using a sandwich ELISA assay, as described previously[5]; an Alexa Fluor® 488-labeled goat anti-mouse IgG secondary antibody was employed in place of the HRP-modified primary antibody used by Kovacs, *et al*.

VLPs were fluorescently-labeled, per manufacturer's instructions, with Alexa Fluor® 555 hydrazide (in conjunction with EDC) for the experiments depicted in Figures 2B-C, 3, 4, and 5A-C, with Alexa Fluor® 555 carboxylic acid succinimidyl ester for the experiments depicted in Figure 5D, and with Alexa Fluor® 532 carboxylic acid succinimidyl ester for the experiment depicted in Figure 2A. To determine the average number of SP94-targeted VLPs internalized by Hep3B and hepatocytes, the capsid was labeled with Alexa Fluor® 488 hydrazide (in conjunction with EDC) and pHrodo™ succinimidyl ester per manufacturer's instructions.

Determination of Dissociation Constants

Adherent and non-adherent cells were grown in suspension flasks to 70–80% confluence or $2–4 \times 10^6$ cells/mL, respectively. Adherent cells were harvested *via* gentle shaking in 5 mM EDTA (diluted in D-PBS) for 30 minutes at 37°C. Cells were counted (Cellometer® automated cell counter; Nexcelom Biosciences; Lawrence, MA), and 1×10^6 cells/mL were placed in siliconized tubes, centrifuged at 4000 rpm for 2 minutes, washed twice with 1X D-

PBS, and re-suspended in complete growth medium. Increasing concentrations of monovalent peptides (labeled with Alexa Fluor® 555 C_2 maleimide) or VLPs (labeled with Alexa Fluor® 555 hydrazide or Alexa Fluor® 555 carboxylic acid succinimidyl ester) were incubated with 1×10^6 cells/mL for 1 hour at 4°C under gentle agitation. One hour is a sufficient period of time for the number of surface-bound particles to reach equilibrium; SP94 and SP88-targeted VLPs exhibit saturable binding (see Figure 5A). Cells were washed four times with 1X D-PBS, and re-suspended in 1 mL of serum-free DMEM without phenol red. Cell samples were immediately analyzed with a FACSCalibur flow cytometer (Becton Dickinson; Franklin Lakes, NJ) equipped with BD CellQuest™ software, version 5.2.1. Data were acquired with the FSC channel in linear mode and all other channels in log mode. Events were triggered based upon forward light scatter, and a gate was placed on the forward scatter-side scatter plot that excluded cellular debris. Samples were excited using the 488-nm laser source, and emission intensity was collected in the FL-2 channel (585/42 filter/bandpass). Mean fluorescence intensity (MFI) was determined using FlowJo Software, version 6.4 (Tree Star, Inc.; Ashland, OR). Cells saturated with SP94-targeted VLPs, monovalent SP94, *etc.* were incubated with ~1 μM of unlabeled SP94 (*i.e.* the saturating concentration of the monovalent peptide) for 1 hour at 4°C, and resulting MFIs were re-measured to determine non-specific binding. Data points that required VLP concentrations of $\geq 1 \times 10^{14}$ particles/mL were collected in 384-well plates using 10–50 μL volumes. Fluorescence emission intensities were determined using a SpectraMax M2e microplate reader (Molecular Devices, Inc.; Sunnyvale, CA); settings were adjusted so that the MFIs of samples exposed to lower VLP concentrations were similar to MFIs collected using flow cytometry. GraphPad Prism (GraphPad Software, Inc.; La Jolla, CA) was used to generate saturation binding curves (ligand or VLP concentration versus MFI), subtract the contribution of non-specific binding (*e.g.* binding of SP94-targeted VLPs in the presence of free SP94) from total binding (*e.g.* binding of SP94-targeted VLPs), and calculate K_d values. GraphPad InStat (GraphPad Software, Inc.; La Jolla, CA) was used to perform the statistical analyses (one-way analysis of variance (ANOVA) and unpaired t-test) summarized in the Figure 5 caption. Plots were generated using Sigma Plot, version 11.0 (Systat Software, Inc.; San Jose, CA).

Determination of the Average Numbers of Internalized VLPs

The average number of SP94-targeted VLPs internalized by each Hep3B or hepatocyte cell was determined using the following procedure. VLPs were labeled, per manufacturer's instructions, with Alexa Fluor® 488 hydrazide (in conjunction with EDC) and pHrodo™ succinimidyl ester after conjugation of the SP94 peptide; pHrodo™ SE becomes more fluorescent upon acidification of its environment, *i.e.* during endocytosis. 1×10^6 cells/mL were exposed to increasing concentrations of particles (1×10^6 to 1×10^{10}) for 1 hour at 4°C, washed, and re-suspended in cold DMEM without phenol red. Samples were analyzed using a FACSCalibur flow cytometer and gated as described above. Alexa Fluor® 488 was excited with the 488-nm laser, and emission intensity was collected in the FL1 channel (530/30 filter/bandpass). Mean fluorescence intensity (MFI) was determined using FlowJo Software, and MFI versus particle concentration data were transformed into Scatchard plots using GraphPad Prism. The average numbers of particles that bind to each Hep3B or hepatocyte cell under saturating conditions were calculated from B_{max} values. Samples were then incubated at 37°C for 1 hour to enable endocytosis of surface-bound particles, and the MFIs of Alexa Fluor® 488 and pHrodo™ SE were measured and plotted against particle concentration; for pHrodo™ SE, the 488-nm laser was used as the excitation source, and emission intensity was collected in the FL2 channel. B_{max} values were determined from Scatchard plots and used to calculate the average number of SP94-targeted VLPs endocytosed by each Hep3B or hepatocyte cell within an hour. Alexa Fluor® 488 was used

to determine the total number of particles associated with each cell, while pHrodo™ was used to determine the number of particles localized within acidic compartments.

Preparation and Analysis of Confocal Fluorescence Microscopy Samples

$1 \times 10^4 – 1 \times 10^6$ cells/mL were seeded on sterile coverslips (25-mm, No. 1.5) coated with 0.01% poly-L-lysine (150–300 kDa) and allowed to adhere for 4–24 hours at 37°C. Cells were incubated with a 5000-fold excess of SP94-targeted VLPs for 15 minutes (Figures 4A and 4B), 30 minutes (Figures 2 and 3), or 60 minutes (Figures 4C and 4D) at 37°C, washed three times with 1X PBS, fixed with 4% formaldehyde (10 minutes at room temperature), and mounted with an anti-fade reagent (SlowFade® Gold). Prior to fixation, cells depicted in Figures 2 and 4 were stained with Hoechst 33342 and either CellTracker™ Green CMDFA (Figures 2B, 2C, and 4A-4D) or CellTracker™ Violet BMQC (Figure 2A) according to manufacturer's instructions. After fixation, cells depicted in Figure 3 were permeabilized with 0.2% Triton X-100 (5 minutes at room temperature) and incubated with a blocking agent (Image-iT FX signal enhancer) for 30 minutes at room temperature. Primary (rabbit pAb against LAMP-1 or mouse mAb against Rab11a) and secondary (Alexa Fluor® 488-labeled goat anti-rabbit IgG or Alexa Fluor® 647-labeled goat anti-mouse IgG, respectively) antibodies were diluted 1:500 in PBS with 1% BSA and incubated with cells for 1 hour at 37°C; cells were counterstained with DAPI prior to mounting.

Three- and four-color images were acquired using a Zeiss LSM510 META (Carl Zeiss MicroImaging, Inc.; Thornwood, NY) operated in Channel mode of the LSM510 software; a 63X, 1.4-NA oil immersion objective was employed in all imaging. Typical laser power settings were: 30% transmission for the 405-nm diode laser, 5% transmission (60% output) for the 488-nm Argon laser, 100% transmission for the 543-nm HeNe laser, and 85% transmission for the 633-nm HeNe laser. Gain and offset were adjusted for each channel to avoid saturation and were typically maintained at 500–700 and −0.1, respectively. 8-bit z-stacks with 1024 × 1024 resolutions were acquired with a 0.7 to 0.9-μm optical slice. LSM510 software was used to overlay channels and to create 3D projections of z-stack images. Images in Figures 2B, 2C, 3, and 4 are collapsed projections. Pearson's correlation coefficients were determined using SlideBook software (Intelligent Imaging Innovations, Inc.; Philadelphia, PA).

The seven-color image (Figure 2A) was acquired using a Zeiss LSM510 META operated in Lambda mode of the LSM510 software; a 63X, 1.4-NA objective was used. Spectral information was acquired over the entire range of the system (411.3-nm to 753.7-nm with a 10.7-nm step) and collected across the 32 PMTs. Laser power and gain were adjusted to avoid saturating the brightest components of the sample. The following settings were used to acquire 8-bit, 1024 × 1024 z-stacks: 14% transmission for the 405-nm diode laser, 3% transmission (60% output) for the 488-nm Argon laser, 3% transmission for the 543-nm HeNe laser, and 3% transmission for the 633-nm HeNe laser; the gain was 850, the offset was −0.15, the optical slice was 0.10 μm, and the frame size was 100 $\mu m.^2$ Control spectra used for unmixing were acquired using singly-labeled control samples. Images were unmixed using the advanced linear unmixing algorithm of the LSM510 software. The brightness and contrast of all channels in the unmixed images were adjusted equally (using the LSM510 software) to balance the intensity between channels. The image in Figure 2A is a collapsed projection.

Cytotoxicity of Drug-Loaded VLPs

MDR was induced in parental Hep3B *via* exposure to increasing concentrations of DOX (25 nM, 50 nM, 75 nM, 100 nM, 150 nM, 200 nM, and 250 nM) in 24-hour intervals, interspersed with 48-hour recovery periods during which cells were incubated in complete

growth medium without DOX.[41] MDR1$^+$ Hep3B cells were exposed to 1 μM of CsA for 72 hours to reverse Pgp-mediated resistance to DOX.[21]

The concentrations of DOX and DOX-loaded VLPs necessary to kill 50% of MDR1$^+$ Hep3B (LC_{50} values – see Figure 6A) were determined by continually exposing 1×10^6 cells/mL to various DOX concentrations for 24 hours at 37°C; the same procedure was used to determine LC_{50} values for free cisplatin and 5-FU. LC_{50} values of the DOX, cisplatin, and 5-FU cocktail and of VLPs loaded with the drug cocktail were determined by continually exposing MDR1$^+$ Hep3B (1×10^6 cells/mL) to various DOX concentrations, in the presence of an equimolar amount of cisplatin and a 3-fold molar excess of 5-FU, for 24 hours at 37°C. Cells were washed three times in 1X PBS (cells that remained adherent were harvested *via* gentle shaking in 5 mM EDTA for 30 minutes at 37°C) and stained with SYTOX® Green nucleic acid stain and Alexa Fluor® 647-labeled annexin V per manufacturer's instructions. The numbers of viable (double-negative) and non-viable (single- or double-positive) cells were determined using a FACSCalibur flow cytometer. SYTOX® Green fluorescence was excited by the 488-nm laser and collected in the FL-1 channel (530/30 filter/bandpass), while Alexa Fluor® 647 fluorescence was excited by the 633-nm laser and collected in the FL-3 channel (670-nm long pass filter). Curves of DOX concentration versus the percentage of viable cells were generated, and GraphPad Prism (GraphPad Software, Inc.; La Jolla, CA) was used to calculate LC_{50} values. The data depicted in Figure 6B was collected by continually exposing 1×10^6 cells/mL of MDR1$^+$ Hep3B or hepatocytes to 285 nM of DOX, the drug cocktail, DOX-loaded VLPs, or VLPs loaded with the cocktail for 24 hours or 7 days at 37°C; the percentage of viable cells in each population was determined as described above. Plots were generated using Sigma Plot, version 11.0 (Systat Software, Inc.; San Jose, CA).

Cytotoxicity of siRNA-Loaded VLPs

The time-dependent viability of Hep3B and hepatocytes exposed to SP94-targeted, siRNA-loaded VLPs (Figure 7A) was determined by exposing 1×10^6 cells to a 1000-fold excess of VLPs for various periods of time (0, 12, 24, 36, 48, 72, 96, and 120 hours) at 37°C. VLPs were loaded with a siRNA cocktail that silences expression of cyclin A2, cyclin B1, cyclin D1, and cyclin E1; 1.1×10^9 particles/mL of VLPs corresponds to a total siRNA concentration of ~150 pM. Cells were washed three times in 1X PBS to remove excess VLPs (cells that remained adherent were harvested *via* gentle shaking in 5 mM EDTA for 30 minutes at 37°C) and stained with Alexa Fluor® 488-labeled annexin V and propidium iodide per manufacturer's instructions. The numbers of cells in the early (positive for annexin V) and late (double-positive for annexin V and propidium iodide) stages of apoptosis were determined using a FACSCalibur flow cytometer. Alexa Fluor® 488 fluorescence was excited by the 488-nm laser and collected in the FL-1 channel (530/30 filter/bandpass), and propidium iodide fluorescence was excited by the 488-nm laser and collected in the FL-2 channel (585/42 filter/bandpass). The total number of apoptotic cells was obtained by adding the numbers of cells in the early and late stages of apoptosis.

The numbers of proliferating and G_0/G_1 arrested Hep3B cells (Figure 7B) were determined by first exposing 1×10^6 cells to SP94-targeted, siRNA-loaded VLPs for various periods of time (same as above) at 37°C; VLPs were loaded with a siRNA cocktail specific for cyclin A2, cyclin D1, and cyclin E1, and the total siRNA concentration was maintained at ~150 pM. Cells were washed three times in 1X PBS to remove excess VLPs. To determine the percentage of proliferating Hep3B, VLP-treated cells were incubated with 10 μM BrdU (in complete growth medium) for 12 hours at 37°C, harvested by gentle shaking in 5 mM EDTA for 30 minutes at 37°C, and fixed with 4% formaldehyde for 30 minutes at 4°C. Cells were then washed three times in 1X PBS with 0.1% Triton X-100; incubated in 1 N HCl for 10 minutes on ice; incubated in 2 N HCl for 10 minutes at room temperature and then 20

minutes at 37°C; incubated in 0.1 M borate for 12 minutes at room temperature; and washed three times in 1X PBS with 0.1% Triton X-100. Cells were blocked in 1X PBS with 0.1% Triton X-100, 1 M glycine, and 5% goat serum for one hour at room temperature and then incubated with an Alexa Fluor® 488-labeleled mouse monoclonal antibody to BrdU (1:100 dilution in 1X PBS with 1% BSA) overnight at 4°C. Cells were washed three times with 1X PBS, and the number of cells positive for BrdU incorporation was determined using a FACSCalibur flow cytometer. Cells were considered positive if their mean fluorescence intensities (MFI) were 100 fluorescence units (FU) greater than the MFI of unlabeled cells. To determine the percentage of G_0/G_1 arrested Hep3B, VLP-treated cells were incubated with 1 μg/mL of Hoechst 33342 for 15 minutes at 37°C, washed three times with 1X PBS, and immediately analyzed using a MoFlo High Performance Cell Sorter (Dako-Cytomation; Carpinteria, CA) equipped with Dako-Cytomation's SUMMIT software, version 4.3.01. Cells were detected using a 488-nm Innova 90 laser (Coherent Inc.; Santa Clara, CA), and a gate was placed on the forward scatter-side scatter plot that excluded cellular debris. Hoechst 33342 was excited with a 355-nm Innova 90 laser, and emission intensity was collected in the FL-6 channel (450/65 filter/bandpass). Single cells were gated using width and area parameters; the area parameter histogram was used to determine the percentage of gated cells in G_0/G_1, S, and G_2/M phases. Data were acquired with the SSC channel in log mode and all other channels in linear mode.

The concentrations of siRNA-loaded VLPs necessary to silence 90% of cyclin A2, cyclin B1, cyclin D1, and cyclin E1 protein expression (IC_{90} values – see Figure 7C for dose-response curves) were determined by continually exposing 1×10^6 cells/mL to various concentrations ($7.1 \times 10^6 – 7.1 \times 10^{10}$ particles/mL, which corresponds to 1 pM – 10 nM of siRNA) of VLPs, loaded with a single type of siRNA, for 48 hours at 37°C. Cells were washed three times with 1X PBS to remove excess VLPs, harvested *via* gentle shaking in 5 mM EDTA (30 minutes at 37°C), fixed with 4% formaldehyde (10 minutes at room temperature), permeabilized with 0.2% Triton X-100 (5 minutes at room temperature), and exposed to a blocking buffer (1X PBS with 5% BSA) for 1 hour at 37°C. Mouse monoclonal antibodies against human cyclin A2, cyclin B1, cyclin D1, and cyclin E1 (1 μg/μL) were labeled with Alexa Fluor® 488 per manufacturer's instructions, diluted 1:500 in 1X PBS with 1% BSA, and incubated with permeabilized cells overnight at 4°C. Cells were washed three times with 1X PBS and analyzed using a FACSCalibur flow cytometer. Initial concentrations of cyclin A2, cyclin B1, cyclin D1, and cyclin E1 were determined by staining untreated cells (*i.e.* not previously exposed to siRNA) with anti-cyclin antibodies, as described above; the percentages of initial cyclin concentrations were then determined by dividing the MFIs of siRNA-treated cells by the MFI of untreated cells. GraphPad Prism (GraphPad Software, Inc.; La Jolla, CA) was used to calculate IC_{90} values from the dose-response curves shown in Figure 7A. Lipoplexes composed of cyclin-specific siRNA and the commercially-available transfection reagent, Lipofectamine™ RNAiMAX (LFA), were prepared according to manufacturer's instructions and employed as controls. Empty SP94-targeted VLPs, non-targeted, siRNA-loaded VLPs, and siRNA alone were also tested for their ability to silence expression of various cyclins in Hep3B and hepatocytes (see Figure 8). The time-dependent decrease in cyclin A2, cyclin B1, cyclin D1, and cyclin E1 concentrations induced by siRNA-loaded, SP94-targeted VLPs (Figure 7D) was determined by incubating 1×10^6 Hep3B cells with a 1000-fold excess of VLPs (1.1×10^9 particles/mL of VLPs ~ 150 pM of siRNA) for various periods of time (0, 12, 24, 36, 48, 72, 96, and 120 hours) at 37°C; VLPs were loaded with a single type of siRNA. Cells were washed, fixed, permeabilized, and stained with Alexa Fluor® 488-labeled anti-cyclin antibodies as described above. The dose- and time-dependent decreases in cyclin A2 mRNA (Figures 7C and 7D, respectively) were determined by incubating Hep3B cells with SP94-targeted VLPs, loaded with the cyclin A2-specific siRNA, as described above. Cells were washed three times with cold 1X PBS to remove excess VLPs; mRNA was isolated from cells and

converted to cDNA using the TaqMan® Fast Cells-to-CT™ Kit. Quantitative PCR was performed by SeqWright, Inc. (Houston, TX). Plots were generated using Sigma Plot, version 11.0 (Systat Software, Inc.; San Jose, CA).

Cytotoxicity of Ricin Toxin A-Chain (RTA)-Loaded VLPs

The percentages of Hep3B and hepatocytes that become positive for caspase-3 activation upon exposure to RTA-loaded, SP94-targeted VLPs were determined by continually incubating 1×10^6 cells/mL with either (1) various concentrations of VLPs (2.0×10^4 – 2.0×10^{10} particles/mL, which corresponds to 1 fM – 1 nM of RTA) for 48 hours at 37°C (Figure 9A) or (2) 2.0×10^6 VLPs ([RTA] = ~100 fM) for various periods of time (0, 12, 24, 36, 48, 72, 96, or 120 hours) at 37°C (Figure 9B). Cells were washed three times with 1X PBS to remove excess VLPs (cells that remained adherent were harvested *via* gentle shaking in 5 mM EDTA for 30 minutes at 37°C), stained with a CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit per manufacturer's instructions, and immediately analyzed using a FACSCalibur flow cytometer. Fluorescein fluorescence was excited by the 488-nm laser and collected in the FL-1 channel (530/30 filter/bandpass). Cells were considered positive for caspase-3 activation if their mean fluorescence intensities (MFIs) were more than 100 fluorescent units greater than the MFI of untreated cells (stained with the CaspGLOW™ kit as described above). RTA-loaded VLPs modified with the SP94 peptide alone or with both the SP94 and H5WYG peptides, as well as RTA alone were employed as controls (Figure 10B). Cells were treated with chloroquine (20 μg/mL) for 24 hours at 37°C to inhibit lysosomal acidification.

The dose- and time-dependent decrease in protein biosynthesis that results upon incubation of Hep3B or hepatocytes with RTA-loaded, SP94-targeted VLPs was determined by continually exposing 1×10^6 cells/mL to either (1) various concentrations of VLPs (2.0×10^4 – 2.0×10^{10} particles/mL, which corresponds to 1 fM – 1 nM of RTA) for 48 hours at 37°C (Figure 9C) or (2) 2.0×10^6 VLPs ([RTA] = ~100 fM) for various periods of time (0, 12, 24, 36, 48, 72, 96, or 120 hours) at 37°C (Figure 9D). Cells were washed three times with 1X PBS to remove excess VLPs, harvested *via* gentle shaking in 5 mM EDTA (30 minutes at 37°C), stained with a Click-iT® AHA Alexa Fluor® 488 Protein Synthesis Assay per manufacturer's instructions, and immediately analyzed using a FACSCalibur flow cytometer. Alexa Fluor® 488 fluorescence was excited by the 488-nm laser and collected in the FL-1 channel (530/30 filter/bandpass). Initial levels of nascent protein synthesis were determined by staining untreated cells with the Click-iT® assay as described above. RTA-loaded VLPs modified with the R8 peptide, empty VLPs modified with either SP94 or R8, non-targeted, RTA-loaded VLPs, and RTA alone were used as controls (Figure 10A). GraphPad Prism (GraphPad Software, Inc.; La Jolla, CA) was used to calculate IC_{90} values from the dose-response curves shown in Figure 9C. Plots were generated using Sigma Plot, version 11.0 (Systat Software, Inc.; San Jose, CA).

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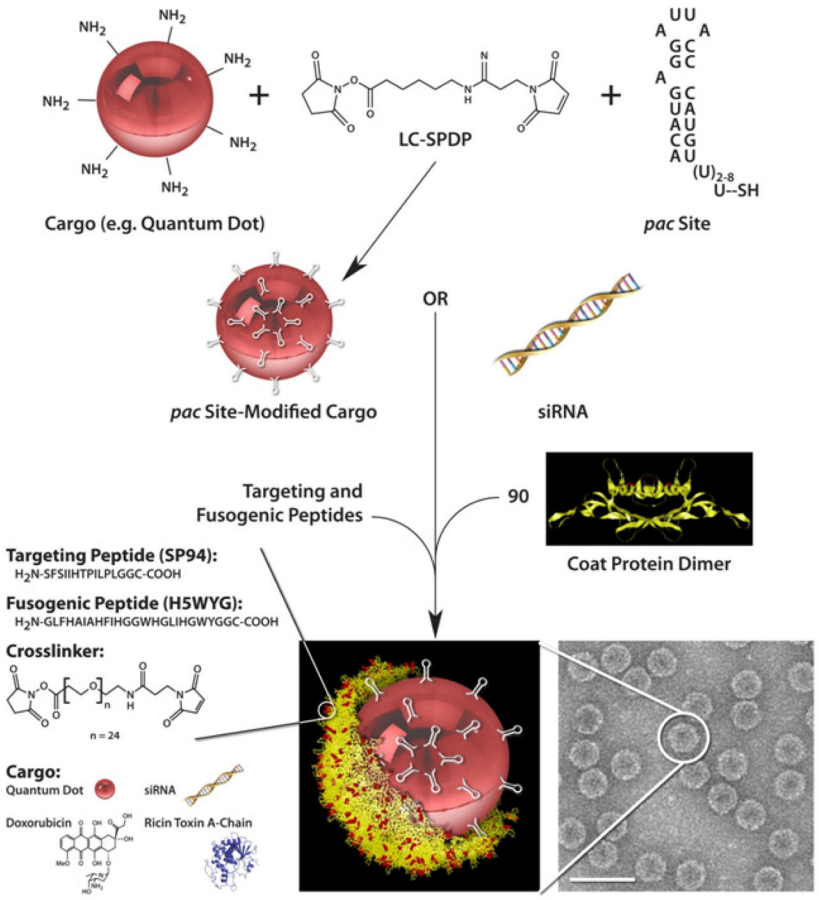

Figure 1. Schematic depicting the process used to synthesize HCC-specific MS2 VLPs that encapsidate chemically disparate therapeutic and imaging agents

Nanoparticles (*e.g.* quantum dots), protein toxins (*e.g.* ricin toxin A-chain), and drugs (*e.g.* doxorubicin) are first conjugated to the *pac* site using an appropriate crosslinker; for example, quantum dots encapsulated within an amine-terminated PEG layer are linked to a derivative of the *pac* site that contains a 3′ uracil spacer and sulfhydryl group using the amine-to-sulfhydryl crosslinker, LC-SPDP. Ninety coat protein dimers then self-assemble around RNA-modified cargo to form the 27.5-nm capsid. siRNA molecules drive capsid re-assembly in the absence of the *pac* site and become incorporated within VLPs at an average concentration of ~85 siRNAs per particle; the yield of fully re-assembled, siRNA-loaded capsids is depicted in the TEM image (scale bar = 50 nm). Cargo-loaded VLPs can be further modified with targeting peptides to promote selective internalization by cancer cells, with fusogenic peptides to promote endosomal escape of internalized VLPs, and with PEG to reduce non-specific interactions and mitigate the humoral immune response against coat protein. Peptides synthesized with a C-terminal cysteine residue are linked to lysine residues (red) on the exterior capsid surface (yellow) *via* a heterobifunctional crosslinker with a PEG spacer arm.

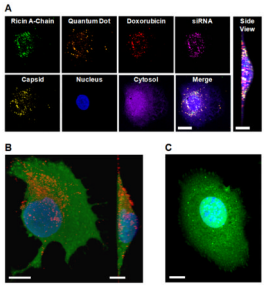

Figure 2. SP94-targeted MS2 VLPs can selectively deliver disparate therapeutic and imaging agents to human HCC

(A) Hyperspectral confocal fluorescence image demonstrating that SP94-targeted VLPs (labeled with Alexa Fluor® 532) can simultaneously deliver ricin toxin A-chain (labeled with Alexa Fluor® 488), quantum dots (Qdot® 585 ITK™ amino(PEG)), doxorubicin (naturally emits at 560–590 nm), and siRNA (labeled with Alexa Fluor® 647) to Hep3B. Cells were labeled with a blue fluorescent nuclear stain (Hoechst 33342) and a purple fluorescent cytosolic stain (CellTracker™ Violet BMQC). Scale bars = 20 μm. **(B) and (C)** Confocal fluorescence microscopy images demonstrating that SP94-targeted VLPs (red) are internalized by Hep3B (B) but not by hepatocytes (C). VLPs were labeled with Alexa Fluor® 555. Cells were labeled with Hoechst 33342 and a green fluorescent cytosolic stain (CellTracker™ Green CMDFA). Scale bars = 10 μm. VLPs were modified with an average of 60 SP94 peptides/particle and were incubated with cells for 30 minutes at 37°C in all experiments.

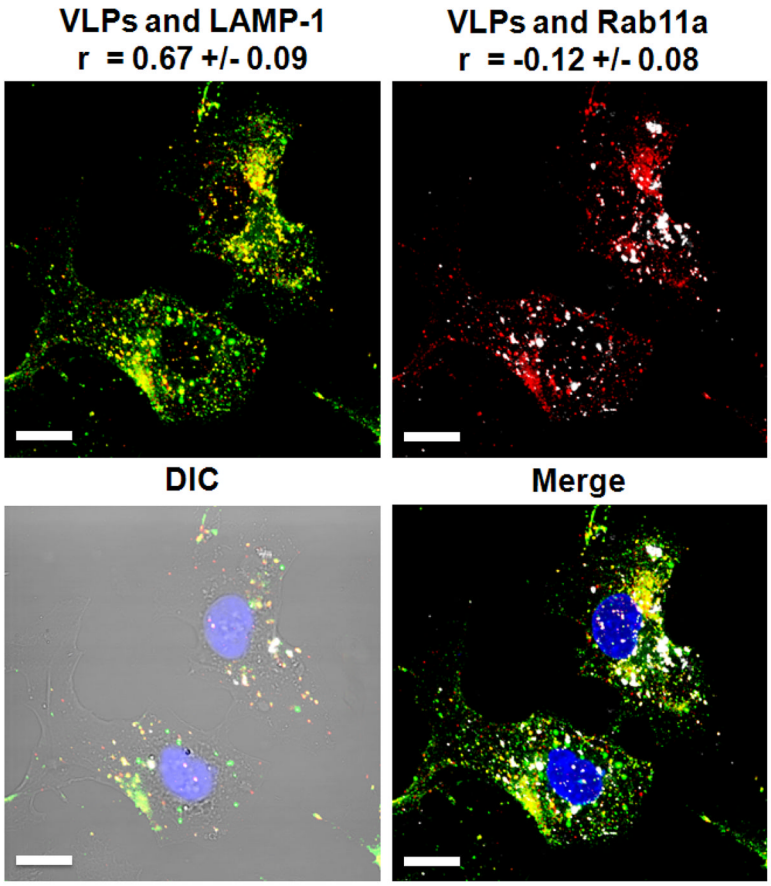

Figure 3. SP94-targeted VLPs are directed to lysosomes upon endocytosis by HCC

Confocal fluorescence microscopy image demonstrating co-localization between Alexa Fluor® 555-labeled VLPs (red) and an Alexa Fluor® 488-labeled marker for lysosomes (LAMP-1, green) but not between VLPs and an Alexa Fluor® 647-labeled marker for recycling endosomes (Rab11a, white). SlideBook software was used to determine Pearson's correlation coefficients (r), which are expressed as the mean value ± the standard de*via*tion for n = 3 × 50 cells. Differential Interference Contrast (DIC) images were employed to define the boundaries of Hep3B cells so that pixels outside of the cell boundaries could be disregarded when calculating r-values. VLPs were modified with an average of 60 SP94 peptides/particle and were incubated with cells for 30 minutes at 37°C. Cells were counter-stained with DAPI. Scale bars = 10 μm.

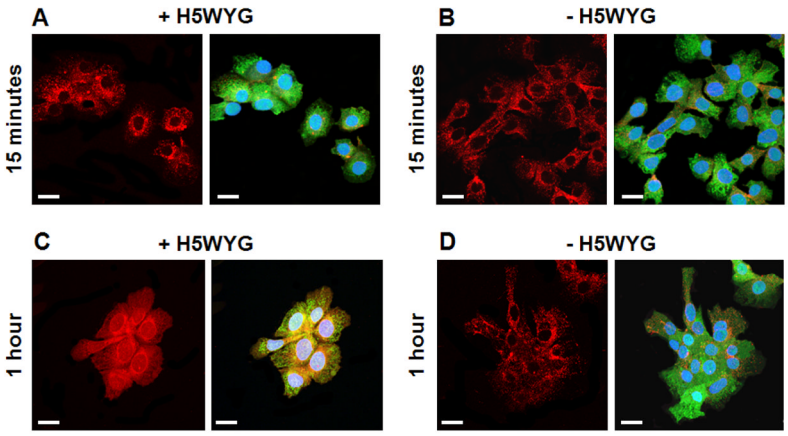

Figure 4. Upon endocytosis, VLPs co-modified with the SP94 targeting peptide and the H5WYG fusogenic peptide become distributed in the cytosol of Hep3B cells, while VLPs modified with just SP94 remain localized in endosomes

(A) – (D) Confocal fluorescence microscopy images of Hep3B cells exposed to SP94-targeted VLPs (red) for either 15 minutes (A and B) or 1 hour (C and D) at 37°C. VLPs were co-modified with ~60 SP94 peptides/particle and ~75 H5WYG peptides/particle in (A) and (C) and with ~60 SP94 peptides/particle alone in (B) and (D). VLPs were labeled with Alexa Fluor® 555. Cells were labeled with Hoechst 33342 and CellTracker™ Green CMDFA. Scale bars = 10 μm.

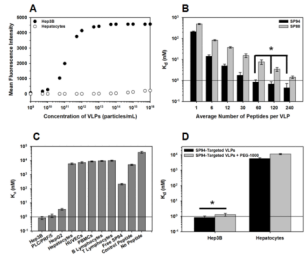

Figure 5. SP94-targeted VLPs have a high specific avidity for HCC, and the degree to which they selectively bind to HCC over hepatocytes can be modulated by peptide density and PEGylation

(A) Sample saturation binding curves for SP94-targeted VLPs (~60 peptides/particle) when exposed to Hep3B ($K_d = 5.1 \times 10^{11}$ particles/mL = 0.85 nM) or hepatocytes ($K_d = 3.5 \times 10^{15}$ particles/mL = 5.8 μM). Saturation binding curves were used to calculate dissociation constants (K_d), which are inversely related to specific avidity. **(B)** K_d values for VLPs modified with various densities of SP94 or SP88 targeting peptides when exposed to Hep3B. * indicates values are NOT significantly different (using one-way ANOVA, $P > 0.05$ for n = 5). **(C)** K_d values for SP94-targeted VLPs (~60 peptides/particle) when exposed to HCC cells (Hep3B, PLC/PRF/5, and HepG2), hepatocytes, endothelial cells (HUVECs), and immune cells (PBMCs and B- and T-lymphocytes). The K_d values of free SP94, VLPs modified with a control peptide that has no known affinity for HCC, and unmodified VLPs (no peptide) when exposed to Hep3B are also given. **(D)** K_d values for VLPs, modified with the SP94 peptide alone (~60 peptides/particle) or with the SP94 peptide (~60 peptides/particle) and PEG-1000 (~145 molecules/particle) when exposed to Hep3B and hepatocytes. * indicates that values are NOT significantly different (using the unpaired t-test, $P > 0.05$ for n = 5). Cell concentrations were maintained at 1×10^6 cells/mL in all experiments. All error bars represent 95% confidence intervals (1.96 σ) for n = 5.

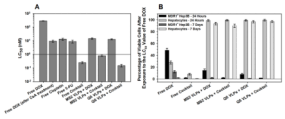

Figure 6. SP94-targeted VLPs of MS2 and structurally-related bacteriophages (*e.g.* Qβ) can deliver a sufficient concentration of chemotherapeutic agents to kill drug-resistant HCC without substantially affecting the *via*bility of hepatocytes

(A) The concentrations of doxorubicin (DOX), cisplatin, 5-fluorouracil (5-FU), a drug cocktail (DOX, cisplatin, and 5-FU), DOX-loaded VLPs, and VLPs loaded with the drug cocktail that are necessary to kill 50% of Hep3B with an induced $MDR1^+$ phenotype (LC_{50} values) within 24 hours at 37°C. $MDR1^+$ Hep3B were exposed to cyclosporin A (CsA) to reverse Pgp-mediated resistance to DOX. **(B)** The percentage of $MDR1^+$ Hep3B and hepatocytes that remain *via*ble upon continual exposure to 285 nM of free drugs or drug-loaded VLPs for either 24 hours or 7 days at 37°C; 285 nM is the LC_{50} value of free DOX when exposed to $MDR1^+$ Hep3B. MS2 and Qβ VLPs were modified with SP94 (~60 peptides/particle) and PEG-1000 (~145 molecules/particle), and cell concentrations were maintained at 1×10^6 cells/mL in all experiments. All error bars represent 95% confidence intervals (1.96 σ) for n = 3.

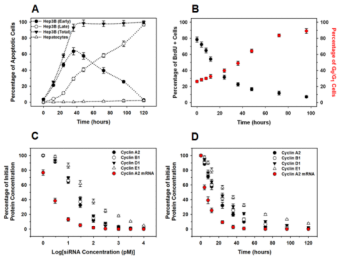

Figure 7. MS2 VLPs are naturally suited for RNA delivery, and modification of the capsid with SP94 enables targeted delivery of siRNA cocktails that silence expression of various cyclins, causing rapid growth arrest and apoptosis of HCC at picomolar concentrations

(A) The percentage of Hep3B and hepatocytes that become apoptotic upon continual exposure to SP94-targeted, siRNA-loaded VLPs for various periods of time at 37°C. VLPs were loaded with a siRNA cocktail that silences expression of cyclin A2, B1, D1, and E1; the total siRNA concentration was maintained at 150 pM. Cells positive for Alexa Fluor® 647-labeled annexin V were considered to be in the early stages of apoptosis, while cells positive for both annexin V and SYTOX® Green, a cell-impermeant nucleic acid stain, were considered to be in the late stages of apoptosis. **(B)** The percentage of Hep3B cells that become arrested upon continual exposure to SP94-targeted, siRNA-loaded VLPs for various periods of time at 37°C. VLPs were loaded with a siRNA cocktail that silences expression of cyclin A2, D1, and E1; the total siRNA concentration was 150 pM. The number of proliferating cells was determined by immunofluorescence-based detection of BrdU incorporation, and the number of cells arrested in G_0/G_1 was determined *via* Hoechst 33342 staining. **(C)** The dose-dependent decrease in cyclin A2, B1, D1, and E1 protein expression upon continual exposure of Hep3B to various concentrations of SP94-targeted, siRNA-loaded VLPs for 48 hours at 37°C. The dose-dependent decrease in cyclin A2 mRNA is included for comparison. **(D)** The time-dependent decrease in cyclin A2, B1, D1, and E1 protein expression upon continual exposure of Hep3B to SP94-targeted, siRNA-loaded VLPs ([siRNA] = 150 pM) at 37°C. The time-dependent decrease in cyclin A2 mRNA is included for comparison. For (C) and (D), VLPs were loaded with a single type of siRNA. Cyclin protein concentrations were determined *via* immunofluorescence, and cyclin A2 mRNA concentrations were determined by real-time PCR. VLPs were modified with SP94 (~60 peptides/particle), H5WYG (~75 peptides/particle) and PEG-1000 (~145 molecules/particle) in all experiments. All error bars represent 95% confidence intervals (1.96 σ) for n = 3.

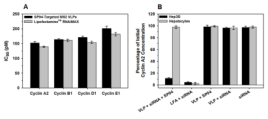

Figure 8. SP94-targeted VLPs loaded with cyclin-specific siRNAs selectively transfect HCC with efficiencies similar to that of commercially-available transfection reagents

(A) The concentrations of siRNA necessary to silence 90% of cyclin A2, B1, D1, or E1 protein expression (IC_{90}) in Hep3B when delivered *via* SP94-targeted VLPs or Lipofectamine™ RNAiMAX (LFA). **(B)** The percentage of initial cyclin A2 protein expression that remains upon exposure of Hep3B and hepatocytes to cyclin A2-specific siRNA using SP94-targeted VLPs and LFA, a non-specific transfection reagent, as delivery vehicles. Empty SP94-targeted VLPs, non-targeted, siRNA-loaded VLPs, and siRNA alone were used as controls. VLPs were modified with SP94 (~60 peptides/particle), H5WYG (~75 peptides/particle) and PEG-1000 (~145 molecules/particle) in all experiments. All error bars represent 95% confidence intervals (1.96 σ) for n = 3.

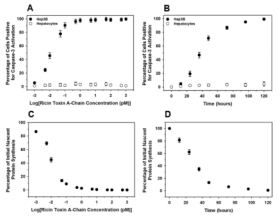

Figure 9. SP94-targeted VLPs that encapsidate ricin toxin A-chain induce apoptosis of HCC at femtomolar concentrations without affecting the *via*bility of hepatocytes

(A) The percentage of Hep3B and hepatocytes that become positive for caspase-3 activation when continually exposed to various concentrations of SP94-targeted, ricin toxin A-chain (RTA)-loaded VLPs for 48 hours at 37°C. **(B)** The time-dependent activation of caspase-3 in Hep3B and hepatocytes upon exposure to SP94-targeted, RTA-loaded VLPs ([RTA] = 100 fM) at 37°C. Caspase-3 activation was quantified using a FITC-labeled derivative of the caspase-3 inhibitor, DEVD-FMK. **(C)** and **(D)** The dose **(C)** and time **(D)** dependent decrease in nascent protein synthesis that was observed upon continual exposure of Hep3B to SP94-targeted, RTA-loaded VLPs at 37°C. Cells were exposed to various concentrations of VLPs for 48 hours in **(C)** and to a fixed concentration of VLPs ([RTA] = 100 fM) for various periods of time in **(D)**. Nascent protein synthesis was quantified using an Alexa Fluor® 488-labeled derivative of methionine. VLPs were modified with SP94 (~60 peptides/particle), H5WYG (~75 peptides/particle), and PEG-1000 (~145 molecules/particle) in all experiments. All error bars represent 95% confidence intervals (1.96 σ) for n = 3.

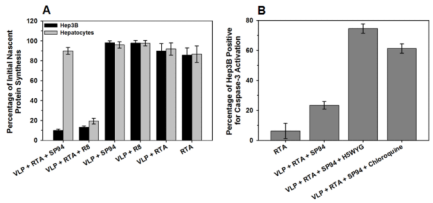

Figure 10. Co-display of SP94 and H5WYG enables RTA-loaded VLPs to become selectively internalized by HCC and to release their cargo into the cytosol before lysosomal conditions destroy the toxin's catalytic activity

(A) The percentage of nascent protein synthesis that remains upon exposure of Hep3B and hepatocytes to RTA-loaded VLPs modified with either the SP94 peptide or with a peptide that promotes non-specific macropinocytosis (R8). Empty SP94-modified VLPs, empty R8-modified VLPs, non-targeted VLPs loaded with RTA, and RTA alone were employed as controls. **(B)** The percentage of Hep3B that become positive for caspase-3 activation when exposed to RTA-loaded VLPs modified with SP94 alone or with a combination of SP94 and H5WYG. Hep3B cells were treated with chloroquine to inhibit lysosomal acidification. Cells were exposed to 100 fM of RTA for 48 hours at 37°C in all experiments. VLPs were modified with PEG-1000 (~145 molecules/particle), as well as an average of ~60 SP94 peptides, ~80 R8 peptides, and/or ~75 H5WYG peptides. All error bars represent 95% confidence intervals (1.96 σ) for n = 3.