The oncolytic adenovirus VCN-01 as therapeutic approach against pediatric osteosarcoma

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None of the authors have potential conflicts of interest to disclose.

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Translational Relevance

Osteosarcoma (OS) is the most common primary malignant tumor of bone in children and adolescents. Despite significant improvements in treatment of the primary tumor, a significant proportion of OS patients eventually develop lung metastases and succumb to their disease even after multistage conventional chemotherapy and surgical excision. Thus, there is a need to develop new and safe approaches for the treatment of OS. Oncolytic adenoviruses have already proven safe and effective in the clinic. VCN-01 is a replication competent adenovirus specifically engineered to replicate in tumors with a defective RB pathway, presents enhanced infectivity through a modified fiber and improved distribution through the expression of a soluble hyaluronidase. Our results show that VCN-01 showed a potent antisarcoma effect meanwhile maintaining a safe toxicity profile in relevant sarcoma animal models. Our results offer a strong preclinical rationale for propelling a phase I/II study with VCN-01 for pediatric osteosarcoma.
Abstract.

Purpose: Osteosarcoma is the most common malignant bone tumor in children and adolescents. Despite aggressive chemotherapy more than 30% of patients do not respond and develop bone or lung metastasis. Oncolytic adenoviruses engineered to specifically destroy cancer cells are one feasible option for osteosarcoma treatment. VCN-01 is a replication competent adenovirus specifically engineered to replicate in tumors with a defective RB pathway, presents an enhanced infectivity through a modified fiber and an improved distribution through the expression of a soluble hyaluronidase. The aim of this study is to elucidate whether the use of VCN-01 would be an effective therapeutic strategy for pediatric osteosarcoma.

Experimental Design: We used osteosarcoma cell lines established from patients with metastatic disease (531MII, 678R, 588M, and 595M) and a commercial cell line (143B). MTT assays were carried out to evaluate the cytotoxicity of VCN-01. Hexon assays were used to evaluate the replication of the virus. Western blot analysis was performed to assess the expression levels of viral proteins and autophagic markers. The antitumor effect of VCN-01 was evaluated in an orthotopic and metastatic osteosarcoma murine animal models.

Results: The current study found that VCN-01, a new generation genetically modified oncolytic adenovirus, administered locally or systemically, had a potent anti-sarcoma effect in vitro and in vivo in mouse models of intra-tibial and lung-metastatic osteosarcoma. Moreover, VCN-01 administration showed a safe toxicity profile.

Conclusion: These results uncover VCN-01 as a promising strategy for osteosarcoma, setting the bases to propel a phase I/II trial for kids with this disease.
Introduction

Osteosarcoma is the most common primary malignant bone tumor (1). It appears principally in the metaphysis of the long bones of children and adolescents (2), often during the period of rapid skeletal growth of the two first decades of life (3). Osteosarcoma is a highly invasive tumor that frequently metastasizes to the lungs. Pathogenesis involves a wide range of different molecular pathways, but of fundamental relevance is the inactivation of tumor suppressor genes, particularly \( p53 \) (4) and the retinoblastoma susceptibility gene (\( RB1 \)) (5, 6); further support for this model has recently come from whole-genome sequencing (WGS) (7).

Nowadays, the standard treatment for osteosarcoma is neoadjuvant chemotherapy followed by conservative surgery (in which, as far as possible, function is preserved), followed by post-operative chemotherapy. Despite aggressive chemotherapy protocols, between 30 to 40% of patients do not respond and relapse, developing bone or lung metastasis (8, 9). For these patients survival remains poor, with an overall five-year survival rate of about 20% (10). Thus, new treatments are needed. Oncolytic adenoviruses engineered to specifically destroy cancer cells are one feasible option.

VCN-01 is an oncolytic adenovirus that harbours a 24-base pair deletion in the E1A region, and the native E1A promoter has been modified by the insertion of eight E2F-binding sites organized in four palindromes and one Sp1-binding site. These modifications selectively restrict the replication of VCN-01 to cells with a defective pRB-pathway (11, 12). In addition, the VCN-01 adenovirus displays improved infectivity and bioavailability, as a result of the inclusion of an RGDK motif in the heparin sulphate-glycosaminoglycans (HSG) binding domain KKTK of the fiber shaft (13); this modification improves antitumor potency compared to the adenovirus modified with a RGD motif in the fiber HI loop (14).
A third modification engineered into the VCN-01 genome is a novel element, an expression cassette with the human PH20 gene (12). This cassette enables expression of a soluble hyaluronidase - active under physiological conditions (pH 7) - that can degrade extracellular matrix hyaluronic acid (HA). HA is a negatively-charged, high molecular weight polysaccharide that forms part of and confers specific properties to the extracellular matrix (15). There is an association between HA and malignancy, and this molecule is overproduced in many cancers (16, 17). In some tumours, high levels of HA have been found to be related with low survival rates and also with development of chemoresistance (18, 19).

Osteosarcoma is characterized by production of malignant osteoid by tumor cells. It has been hypothesized that degradation of HA can destabilize such osteoid thereby improving viral efficacy. Supporting this notion, several studies have shown that drugs capable of disturbing hyaluronan-rich pericellular matrix cells result in less accumulation of hyaluronan (HA), and that, consequently, these drugs have a potent antisarcoma effect in vivo and in vitro (20). It remains to be demonstrated whether VCN-01 expressed-hyaluronidase degrades HA present in the pericellular matrix of tumours, thereby improving viral spread and increasing the oncolytic effect of VCN-01.

In this study, we analysed the antisarcoma effect of VCN-01. Our data show that VCN-01 exerts a potent antitumor effect in vitro and in vivo, in an intratibial and lung metastatic models. These results uncover VCN-01 as a promising strategy for osteosarcoma, setting the bases to propel a phase I/II trial for pediatric osteosarcoma.
Materials and Methods

Cell lines and culture conditions.

Primary osteosarcoma-derived cell lines 531MII, 588M, 595M and 678R were developed at the Clínica Universidad de Navarra as previously described (15). In addition we used the 143B cell line, obtained from the American Type Culture Collection (ATCC, Manassas, VA). All the cell lines were maintained in Minimum Essential Medium (MEM\(\alpha\)) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO\(_2\) at 37 °C. All the cell lines were tested and authenticated at the CIMA Genomic Core Facility using short tandem repeats DNA profiling.

Adenovirus construction and infection.

The construction of VCN-01 and method of viral infection followed procedures described elsewhere (21, 22).

Cell viability assays.

531MII, 588M, 595M and 678R cells were seeded at a density of 1x10\(^3\) cells per well in 96-well plates, and one day later cells were infected with VCN-01 at MOIs (multiplicity of infection) of 5, 10, 20, 40 and 100. Cell viability was assessed five days later using the MTT assay (Sigma-Aldrich) as previously described (23). Dose–response curves were analyzed using GraphPad Software (La Jolla, CA), which fits the dose-response curves to Chou–Talalay lines (24); IC\(_{50}\) is the median-effect dose (the dose causing 50% of cells to be affected, i.e., in this case IC\(_{50}\) is the amount of virus that results in 50% cell-death or, equivalently, 50% survival). In each experiment cells were plated in triplicate and each experiment was performed three times.
**Viral replication assays.**

Osteosarcoma cells were seeded at a density of $1 \times 10^4$ cells/well in six-well plates and infected 20 hours later with 1 MOI of VCN-01. Three days later, cells were collected, and the final amount of virus was determined by means of a method based on anti-hexon staining in HEK293 cells (as described in reference 25).

**Immunoblotting.**

For immunoblotting assays, samples subjected to the same treatment as described earlier, and containing identical amounts of protein, were subjected to sodium dodecyl sulfate–Tris–glycine gel electrophoresis. Membranes were incubated with the following antibodies: E1A, (Santa Cruz Biotechnology, Santa Cruz, CA), fiber (NeoMarkers, Fremont, CA), GRB2 (BD Transduction Laboratories, Frankling Lakes, NJ). The membranes were developed according to Amersham’s enhanced chemolumiscence protocol.

**Real-time quantitative PCR analysis of gene expression.**

Total RNA was isolated and cDNA was synthesized using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Total RNA was extracted from paraffin-embedded sections using RecoverAll Total Nucleic Acid Isolation (Ambion by Life Technologies). Reverse transcriptase reactions contained 1 μg of RNA, 1X RT buffer, 4 μl of dNTPs (100 mM), 1X random primers, 5 μl of MultiScribe Reverse Transcriptase (50 Units/μl) and 0.005 μl of RNase Inhibitor (0.20 units/μl). The reactions were incubated in a GeneAmp PCR System 2400 (Applied Biosystems) for 10 min at 25°C and then 2h at 37°C. Each cDNA sample was analysed in triplicate by fast quantitative-PCR analysis (Applied Biosystems 7900 Sequence Detection System)
using fast SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and specific primers for fiber (forward: 5’-CAAACGCTGTTGGATTTATG-3’; reverse: 5’-GTGTAAGAGGATGTGGCAAAT-3’), PH20 (forward: 5’-TACACACTCCTTGCTTGG-3’; reverse: 5’-CTTAGTCTCAGAGGCCAC-3’), and the house-keeping gene GAPDH (forward: 5’-AGCCACATCGCTCAGACAC-3’; reverse: 5’-GCCCAATACGACCAAATCC-3’). For thermal cycling, fast real time conditions were: 20 s at 95 ºC for one cycle; 1 s at 95 ºC and 20 s at 60 ºC for 40 cycles. To determine relative levels of gene expression, the comparative threshold cycle (Ct) method was used, and data were presented as $2^{-\Delta\Delta Ct}$.

**Animal studies.**

Ethical approval for animal studies was granted by the Animal Ethical Committee of the University of Navarra (CEEA; Comité Etico de Experimentación Animal under the protocol number CEEA/065-13 and CEEA/075-13).

For the orthotopic intra-tibial model, 531MII osteosarcoma cells ($5 \times 10^5$) were engrafted by injection through the tibial plateau in the primary spongiosa of both tibias of female nude mice (Taconic Farms, Inc). Seven days after injection, animals were randomized to 3 groups (control with PBS and two groups of VCN-01 treated with two different doses). PBS or VCN-01 ($10^7$ pfu/animal and $10^8$ pfu/animal) were administered into the tibia of the animals on days 7 and 14 after cell implantation. Tumor development was monitored every week until the end of the experiment (day 90 after cell implantation) and mice were then sacrificed. When a tumor became visible, its size was measured weekly, and tumor volume was calculated (26).

For the lung metastatic model, 531MII osteosarcoma cells ($2 \times 10^6$ cells) were injected through the tail vein. Seven days later animals were arbitrarily randomized to 3 groups.
(control, PBS-treated, or VCN-01 with the same doses as above) and the VCN-01 virus was intravenously administered in the tail vein,

All animals were weighed every two weeks throughout the experiment in order to evaluate toxicity. Animals were weighed weekly during the period of VCN-01 administration.

**PET analyses.**

As part of the orthotopic study, at the end of the experimental procedure, antitumoral effect was measured by positron emission tomography (PET) with the radiotracer 18 fluorodeoxyglucose (\(^{18}\text{F-FDG}\)). Overnight prior to PET, mice were fasted but allowed water. On the day of PET, mice were anesthetized with 2% isoflurane in 100% O\(_2\) and \(^{18}\text{F-FDG}\) (17.7 MBq ± 2.6 in 80-100 μL) was injected into the tail vein. To avoid radiotracer uptake in the hindlimb muscle, \(^{18}\text{F-FDG}\) uptake was performed under continuous anesthesia for 50 min. PET imaging was performed in a dedicated small animal Philips Mosaic tomograph (Cleveland, OH), with 2 mm resolution, 11.9 cm axial field of view (FOV) and 12.8 cm transaxial FOV. Anesthetized mice were placed horizontally on the PET scanner bed to perform a static acquisition (sinogram) of 15 min duration. Images were reconstructed using the 3D Ramla algorithm (a true 3D reconstruction) with two iterations and a relaxation parameter of 0.024 into a 128×128 matrix with a 1 mm voxel size; corrections were applied for dead time, decay and random and scattering corrections. For the assessment of \(^{18}\text{F-FDG}\) uptake, all studies were exported and analyzed using the PMOD software (PMOD Technologies Ltd., Adliswil, Switzerland). Regions of interest (ROIs) were drawn on coronal 1-mm-thick small-animal PET images on consecutive slices including entire hindlimbs. Finally,
maximum standardized uptake value (SUV$_{\text{max}}$) was calculated using the formula SUV = \[
\text{tissue activity concentration (Bq/cm}^3\text{)/injected dose (Bq)} \times \text{body weight (g)}.
\]

**Immunohistochemical analysis.**

Paraffin-embedded sections of mouse leg, lung and liver were immunostained with specific antibodies for adenoviral mouse-hexon (Chemicon International, Inc., Temecula, CA); E1A (Santa Cruz Biotechnology, Santa Cruz, CA); biotinylated hyaluronan binding protein (AMS biotechnology, Oxon, UK); and vimentin clone V9 (IS30, Dako Denmark A/S, Glostrup, Denmark). Conventional procedures were followed in all cases (27). For immunohistochemical staining, Vectastain ABC kits (Vector Laboratories Inc., Burlingame, CA) were used according to the manufacturer’s instructions.

**Statistical analysis.**

Data were expressed as mean ± SD. For the \textit{in vitro} experiments, data were evaluated with the two-tailed Student’s t-test. The same test was used to compare data for treatment and control groups in the \textit{in vivo} experiments. The program Graphpad Prism 5 (Statistical Software for Sciences, La Jolla, CA, USA) was used for the statistical analysis.
Results

Characterization of the in vitro anti-osteosarcoma effect of VCN-01

As mentioned in the introduction, VCN-01 is an improved genetically modified adenovirus that presents in its genome several modifications that renders a more specific and potent virus; E2F binding sites in the E1A promoter, a 24 bp deletion in the E1A gene, modified fiber and finally VCN-01 has an expression cassette inserted in its genome that allows the virus to express PH20 (hyaluronidase) (Fig. 1A).

First, we wanted to evaluate the in vitro antisarcoma effect of VCN-01. To this end, we performed MTT analyses in 4 primary (531 MII, 588M, 595M, 689R) and one established (143B) osteosarcoma cell lines. MTT assays showed that VCN-01 induced cell death in a dose-dependent manner in all cell lines. The IC_{50} of VCN-01 ranged between 1 and 30 MOIs. 531MII was the most sensitive line and 588M the most resistant (Fig. 1B and Supplementary Fig. S1A).

Assessment of viral protein levels in cells previously infected with VCN-01 revealed robust expression of E1A, an early viral protein, and fiber, a late protein. Levels of both proteins followed a dose-dependent pattern (Fig. 1C and Supplementary Fig. S1B). These data suggest that there was effective viral transcription and that the virus was replicating.

To confirm the existence of an effective viral cycle in osteosarcoma cell lines, we used a replication assay based on anti-hexon staining. Viral counts ranged from 10^4 to 10^7 pfu/ml, indicating efficient replication in all cell lines (Fig. 1D and Supplementary Fig. S1C). In 531MII and 678R cell lines, the strength of VCN-01's cytotoxic effect correlated with viral titers.

Finally, we assessed the expression levels of the PH20 produced by the virus and whether those levels were correlated with fiber expression. We performed mRNA
expression analysis and we observed that PH20 and fiber mRNA levels correlated with each other in both cell lines tested ($R^2=0.99$, $p<0.0001$) indicating that VCN-01, when transcribed, can express the PH20 cassette inserted in the viral genome (Fig. 1E and Supplementary Fig. S1D).

Altogether these data indicate that, in pediatric osteosarcoma-derived cell lines, VCN-01 replicated in vitro, expressed the PH20 enzyme and exerted a robust oncolytic effect.

**Evaluation of viral toxicity in vivo**

In order to evaluate the potential toxicity of local and systemic viral administration, weight was controlled every week after local (intratumoral) or systemic (tail vein) viral administration. Mice did not lose weight with VCN-01 administration; route of administration (intratumoral in the intra-tibial model versus tail vein in the lung-metastatic model) did not affect weight loss and neither did viral concentration (Figs 2A and 2B).

Hematoxylin-eosin staining of liver sections did not reveal morphological alterations associated with hepatotoxicity (Figs 2C and 2B). We observed no Councilman bodies, macrosteatosis or necrotic areas. Immunohistochemistry did not detect expression of E1A and hexon (Supplementary Fig.S2A and Fig.S2B) in the liver, indicating absence of virus (Fig. 3B).

Thus, in mice, locally- or systemically- administrated VCN-01 was not significantly toxic and was well tolerated.

**Antitumor effect of VCN-01 in an orthotopic osteosarcoma model**

Next, we analyzed the therapeutic effect of VCN-01 in an orthotopic osteosarcoma model. For that purpose, we inoculated 531MII cells in the tibial tuberosity of nude mice followed by VCN-01 injection at days 7 and 21 administered at
two different dosages (10⁷ pfu/animal or 10⁸ pfu/animal). PBS was administered to the control group (Supplementary Fig. S3A). Tumor development was monitored every week until the end of the experiment (day 90 after cell implantation) and mice were then sacrificed.

By the end of the experiment, there were clearly visible differences in tumors in the three groups of mice (Fig. 3A). PET imaging also showed that tumor volumes were significantly bigger in the control group relative to those in the 10⁸ pfu group (Figs 3B and 3C). All control mice (five mice x 10 tibias) developed visible tibial tumors, while only three out of 10 tibias in the group treated with 10⁷ pfu VCN-01) and none of the mice in the group treated with 10⁸ pfu VCN-01 group did (Figs 3D and 3E). Tumor volumes of control mice ranged between 100 and 500 mm³; volumes for mice in the 10⁷ pfu group were between 50 and 400 mm³.

Pathologically, at the end of the experiment, tumors had the characteristic morphology of osteosarcoma and a notable production of malignant osteoid (Fig. 3F). In some control mice, tumors had grown such that they had crossed the epiphysis of the tibiae, resulting in transarticular tumors. In the VCN-01 lower dose treated group, there was evidence of a certain tumoral burden in some tibiae. Mice in the higher dose treated group showed no evidence of tumor.

At the end of the experiment, approximately 70% of control mice presented lung metastases derived from the primary osteosarcoma tumour (Supplementary Fig. S4B). Of mice treated with VCN-01, only one, in the 10⁷ pfu treated-group, developed lung metastasis (Figs 3E and 3F and Supplementary Figs S4B and S4C).

Hexon staining verified viral presence (Supplementary Fig. S4A). In hexon positive areas, immunohistochemistry indicated decreased HA expression and a lower density of cancer cells. These observations, beyond an indirect demonstration of the
oncolytic effect of VCN-01, suggest that VCN-01 was able to express PH20 and that this PH20 was functional at digesting HA (Supplementary Fig. S4A).

RNA extracted from tibial sections was used to assess the expression of fiber and PH20. Expression of fiber and hyaluronidase mRNA was higher in mice treated with VCN-01 $10^8$pfu group relative to those in the VCN-01 $10^7$pfu treated group (Fig. 3G). As in the in vitro experiments, there was a significant correlation between the levels of expression of hyaluronidase PH20 and fiber mRNA.

In conjunction, these results show that VCN-01 replicated in vivo inside tumors, where it produced functional hyaluronidase and had a potent anti-osteosarcoma effect.

**Therapeutic effect of VCN-01 in a metastatic osteosarcoma model**

Since the lungs are preferred metastasis organs for osteosarcoma we evaluated the anti-tumor effect of VCN-01 in a lung metastatic osteosarcoma model using the cell line 531MII. This cell line recapitulates the pathology found in osteosarcoma lung metastasis in patients. We administrated $2\times10^6$ cells in the tail vein of athymic nude mice and, seven days later, mice were treated with four intravenous injections at doses of $10^7$ pfu/animal of VCN-01 or $10^8$ pfu/animal of VCN-01. Mice were sacrificed at day 60 (Supplementary Fig. S3B). The objective of this experiment was the assessment of tumor burden in mice lungs and the evaluation of the capacity of VCN-01 to reach the tumors and to replicate in tumor cells after systemic administration. This virus has a modification in the fiber that improves its half-life in blood, what might improve the antitumor effect compared to other tested virus.

The pathologic analysis of lung metastases showed the characteristic appearance produced by 531MII. This phenotype was verified by V9 positive staining (Figs 4A and 4C). Lung tumor burden was significantly reduced in both VCN-01-treated groups.
compared to the control group (PBS-treated mice): by the end of the experiment (day 60), a mean of 2.06% of lung surface area in untreated mice presented tumor, while this percentage in the $10^8$ pfu and $10^7$ pfu groups was 0.4 and 0.14%, respectively (Fig. 4B). In addition, mice treated with VCN-01 had less tumor nodules (Fig. 4C).

Viral fiber and PH20 expression in the lungs was analyzed by qRT-PCR, which demonstrated that the virus was able to target and to replicate in tumor cells after systemic administration (Fig. 4D). The overall mRNA expression of viral fiber and PH20 in the lungs of mice treated with VCN-01 $10^7$ pfu/animal was higher than that in mice treated with VCN-01 $10^8$ pfu/animal. This result can be explained by hypothesizing that at $10^8$ pfu/animal VCN-01 was left with few tumoral cells in which to replicate.

In summary, VCN-01 displayed a significant and potent anti-sarcoma effect in the 531MII lung-metastatic osteosarcoma model.
Discussion

Previously, others and ourselves have shown that oncolytic adenoviruses present a potential therapeutic approach for treatment of pediatric osteosarcoma (28-31). The current study found that VCN-01, a new generation genetically modified oncolytic adenovirus, administered locally or systemically, had a potent anti-sarcoma effect \textit{in vitro} and \textit{in vivo} in mouse models of intra-tibial and lung-metastatic osteosarcoma.

VCN-01 was engineered - by inclusion of eight E2F response elements inside its genome and the deletion of 24 bp in the E1A gene - to replicate preferentially in cancer cells with an aberrant RB pathway. We observed VCN-01 to have its strongest antitumor effect in the 531MII cell line, which harbours a Rb mutation. Because Rb mutations are associated with malignance and poor prognosis in osteosarcoma patients (32), that VCN-01 worked well in this scenario is of particular relevance.

Several studies have found a relationship between levels of HA and tumoral resistance to chemotherapy, with associated poor prognosis for patients (17, 18). Therefore, in the treatment of osteosarcoma, VCN-01 can have two therapeutic effects: an intrinsic oncolytic effect on tumor cells and the effect of hyaluronidase on the extracellular matrix (33). In respect of the latter effect, inhibitors of hyaluronic acid retention have been shown to be able to modify the pericellular matrix in an osteosarcoma, restraining tumor formation \textit{in vivo} and also reducing the occurrence of metastasis (34). Additionally, several works have found that hyaluronidase disturbed cell-cell interaction within the tumor mass, thereby enhancing the diffusion of antitumor agents (33, 35). Following this line of thinking, further studies are needed to assess the promising possibility that the complementary effects of VCN-01 and chemotherapy used in conjunction will result in enhanced diffusion of both virus and drugs and an improved anti-sarcoma efficacy.
An important limitation of therapy with systemically administered adenovirus is clearance of virus by the immune system (36). Virtually the entire human population has IgG antibodies to adenovirus serotype 5, and 55% of these antibodies effectively neutralize adenoviruses (37). The insertion of an RGDK sequence into the fiber protein produced by VCN-01 considerably improved its bio-distribution after systemic administration and enhanced the therapeutic effect of VCN-01 in two immune-competent models (11, 38). Currently, there are two clinical trials underway to evaluate the therapeutic effect of systemically administered VCN-01; one of these concerns VCN-01 alone and the other VCN-01 in combination with gentamicine (NTC02045602 and NTC02045589).

In summary, VCN-01 exerted a potent anti-osteosarcoma effect in vitro and in vivo and was safe in terms of toxicity. Our results consolidate the case for pursuing VCN-01 as a realistic therapeutic option for kids with osteosarcoma.
Bibliography


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CONFLICT OF INTEREST

MC and RA are shareholder of VCN Biosciences
Figure legends

Figure 1.

VCN-01 exerts a potent oncolytic effect in pediatric osteosarcoma cell lines, 531MII and in 143B. A, Illustrative scheme of genetic modifications in VCN-01 genome. B, Cell viability analyses of osteosarcoma cell lines after viral treatment. 143B and 531MII cell lines were infected with VCN-01 at the indicated multiplicities of infection (MOIs). Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays 5 days after infection. Data are shown as the percentage (mean ± SD) of cells alive after infection with VCN-01. C, Viral protein expression in osteosarcoma cell lines infected with VCN-01 assessed by western blot. D, Quantification of VCN-01 replication in the indicated cell lines. Viral counts were determined three days after VCN-01 infection at 1MOI by culture infection antihexon staining–based method in HEK293 cells and expressed as plaque forming units (pfu) per millilitre. Data are shown as the mean ± SD of three independent experiments. E, Correlation of viral PH20 and Fiber mRNA expression measured by RQ-PCR in osteosarcoma cell lines previously infected with VCN-01. mRNA levels are presented as $2^{-\Delta\Delta Ct}$ standardized with their constitutive gene and compared to an uninfected control.

Figure 2.

VCN-01 local or systemic administration showed a safe toxicity profile in osteosarcoma models in vivo. A, and B, Body weight plotting of animals treated locally (A) or systemically (B) with VCN-01 at the indicated pfu. Mice from the different groups were weighed every week during treatment. Data is shown as the median ± SD within each group at each time point. C, Histological analysis of mice livers bearing an orthotopic osteosarcoma and treated locally with VCN-01 at $10^8$ pfu. H&E (upper panels,
magnification x400) and E1A immunohistochemistry (lower panels, magnification x400). D, Histological analysis of mice livers bearing a lung metastatic osteosarcoma and treated systemically with VCN-01 at $10^8$ pfu. Representative micrographs of H&E and E1A immunohistochemistry of mice livers from indicated groups of the metastatic osteosarcoma model. Images (x400 magnification) showed no viral presence in mice livers and no signs of hepatotoxicity were found.

**Figure 3.**

VCN-01 antisarcoma effect in the orthotopic osteosarcoma model with the 531MII cell line. Tumors were developed by orthotopic injection of 500,000 531MII cells in the tibial tuberosity of female nude mice and 90 days later were sacrificed. A, Analyses of tumor burden development. Tumor volume in the mice tibias was measured at different days until the end of experiment. B, PET images of tumor burden. Representative images of PET analyses of control mice or treated with VCN-01 ($10^7$ or $10^8$ pfu). C, Quantification of tumor burden by PET with the radiotracer 18 fluorodeoxyglucose ($^{18}$F-FDG). Maximum standardized uptake value (SUVmax) was calculated using the formula SUV = $[\text{tissue activity concentration (Bq/cm}^3/\text{injected dose (Bq)}] \times \text{body weight.}$ Represented are the mean ± SD, SUV values of the tumors of all animals in the same group (Wilcoxon test). D, Representative macroscopic images of mice tibias from all treated groups. E, Tumor volume evaluation. Quantification of tumor volume was calculated by the formula: Volume (mm$^3$) = $[(\text{Long diameter} \times \text{perpendicular diameter}^2)/2]$. Represented are the mean ± SD of each tumor in the same group (Student’s t-test). F, Hematoxylin and eosin staining of tibias from mice of indicated therapy groups. Representative photomicrographs of a control and two animals that received the different dose-protocols of VCN-01 (magnification, x100). G, Quantification of fiber and PH20 mRNA expression of cDNA from orthotopic osteosarcoma tumors by qRT-
PCR and its correlation graph. mRNA levels are presented as $2^{-\Delta\Delta C_t}$ standardized with their constitutive gene and compared to an uninfected control. Data is given as the mean ± SD.

**Figure 4.**

VCN-01 administered systemically reduced osteosarcoma lung metastasis *in vivo*. A, Lung metastases were induced by endovenous injection of $2 \times 10^6$ 531-MII cells in the tail of female nude mice. Animals were randomized as positive control (no treatment), VCN-01 group $10^7$ pfu ($10^7$ pfu/week x 4 weeks) and VCN-01 group $10^8$ pfu ($10^8$ pfu/week x 4 weeks). At day 60 animals were sacrificed. A, Representative H&E photomicrographs at 200x of lung lesions from mice of the three groups described. B, Quantification of lung tumor burden (531-MII) in mice at the end of the experiment. Bar representation of tumor area relative to total lung area. The values represent mean percentage tumor area of animals from each group, as obtained from a specifically designed program that measured both tumor area and total lung area from which was calculated the percentage of tumor area. C, 200x vimentin (V9 antigen) immunohistochemistry images showing metastatic implants in lung of mice from untreated and VCN-01 treated groups. E, Quantification of number of lung tumor nodules. Bar representation of tumor number represent different quantifications of different observers. E, Quantification of fiber and PH20 mRNA expression of cDNA from osteosarcoma tumors by qRT-PCR and its correlation graph. mRNA levels are presented as $2^{-\Delta\Delta C_t}$ standardized with their constitutive gene and compared to an uninfected control.
**Figure 1**

A. Schematic diagram of the viral genome showing the major late promoter (MLP) and the gag-pol genes. E2F binding sites are indicated.

B. Cell proliferation study showing the IC₅₀ values for 143B and 531MII. The IC₅₀ for 143B is 12.5 MOIs, and for 531MII is 1.0 MOIs.

C. Western blot analysis showing the expression of Fiber, E1A, and GRB2 proteins.

D. Viral replication study showing the viral replication levels (pfu/ml) for 143B and 531MII.

E. mRNA expression study showing the levels of PH20 and Fiber mRNAs (2⁻ΔΔCt) for 143B and 531MII. Linear regression analysis with R² values.
Figure 2

(A) Intratibial model

(B) Lung metastatic model

(C) HE

(D) IHC E1A

Control 10⁷pfu VCN-01

Control 10⁸pfu VCN-01
Figure 4

A) Tumor Area/Total Area

B) PH20 mRNA (2^-ΔΔCT)

C) Number of tumor nodules

D) Fiber mRNA (2^-ΔΔCT)

E) VCN-01
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