Safety and efficacy of VCN-01, an oncolytic adenovirus combining fiber HSG-binding domain replacement with RGD and hyaluronidase expression

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ABSTRACT

PURPOSE: Tumor targeting upon intravenous administration and subsequent intratumoral virus dissemination are key features to improve oncolytic adenovirus therapy. VCN-01 is a novel oncolytic adenovirus that combines selective replication conditional to pRB pathway deregulation, replacement of the heparan sulfate glycosaminoglycan putative-binding site KKTK of the fiber shaft with an integrin-binding motif RGDK for tumor-targeting, and expression of hyaluronidase to degrade the extracellular matrix. In this study we evaluate the safety and efficacy profile of this novel oncolytic adenovirus.

EXPERIMENTAL DESIGN: VCN-01 replication and potency was assessed in a panel of tumor cell lines. VCN-01 tumor-selective replication was evaluated in human fibroblasts and pancreatic islets. Preclinical toxicity, biodistribution, and efficacy studies were conducted in mice and Syrian hamsters.

RESULTS: Toxicity and biodistribution preclinical studies support the selectivity and safety of VCN-01. Antitumor activity after intravenous or intratumoral administration of the virus was observed in all tumor models tested, including melanoma and pancreatic adenocarcinoma, both in immunodeficient mice and immunocompetent hamsters.

CONCLUSION: Oncolytic adenovirus VCN-01 characterized by the expression of hyaluronidase and the RGD shaft retargeting ligand shows an efficacy-toxicity profile in mice and hamsters by intravenous and intratumoral administration that warrants clinical testing.
TRANSLATIONAL RELEVANCE

Oncolytic adenoviruses are promising agents for cancer therapy. However, clinical experience points the need to increase antitumor efficacy. Inefficient tumor targeting after systemic administration and poor intratumor dissemination of the oncolytic adenovirus are key factors to improve. In this work, we address both of these limitations by combining a tumor targeting capsid modification and the expression of the extracellular matrix degrading enzyme hyaluronidase in a single oncolytic adenovirus named VCN-01. We report a good safety and efficacy profile of this virus in two different animal models, mice and hamster. We think that this novel oncolytic adenovirus could contribute to improve clinical outcome of cancer patients in which standard treatments have failed. The results here presented have supported the recent initiation of two Phase I clinical trials in patients with pancreatic cancer or other tumor types.
INTRODUCTION

The use of viruses to treat cancer is an old concept that has been revisited during the last two decades with viruses genetically modified to acquire selectivity and potency. Currently, genetically modified herpes simplex virus, vaccinia viruses, and adenoviruses are in Phase III clinical trials. GM-CSF expression by these viruses seeks to elicit antitumor immunotherapy(1, 2). Despite that this immune mechanism of action is expected to be systemic, the strong local immunosuppressive tumor environment may require that the virus reaches and replicates in all tumor nodules for effectiveness(3). Accordingly, it would be beneficial to improve systemic tumor targeting and intratumoral dissemination of oncolytic viruses.

Poor adenovirus tumor targeting upon intravenous administration has been associated with multiple neutralizing interactions in blood, tropism for liver and spleen, and clearance by macrophages(4). Different capsid modifications are being explored in order to avoid liver transduction and to expose specific ligands for tumor cells(5). The mutation of the putative heparan sulfate-glycosaminoglycans (HSG) binding domain KKTK, located in the fiber shaft, abrogates liver transduction in mice, rats, and non-human primates(6-8). However, the insertion of targeting peptides in the HI loop of these shaft-modified fibers does not rescue viral infection of tumor cells(9, 10). Our group previously described that the replacement of the KKTK domain with an RGD motif, significantly increased tumor cell transduction and improved the tumor-to-liver ratio in vivo in the context of a non-replicative adenovirus(11). Moreover, when incorporated in an oncolytic adenovirus background, the RGDK modification resulted in increased bioavailability after systemic administration and, consequently, in better antitumor efficacy when compared with a virus containing the RGD motif in the HI loop of the fiber(12).

Regarding intratumoral dissemination, the extracellular matrix (ECM) has a prominent role at inhibiting viral spread, acting as a physical barrier and raising the interstitial fluid pressure
(IFP) in tumors(13-15). To tackle this problem, oncolytic viruses have been armed with ECM-degrading enzymes such as relaxin, decorin, metalloprotease-9, chondroitinase ABC(16-20), or PH20 hyaluronidase as we have previously reported(21). Additionally, recombinant hyaluronidase enhances the penetration and efficacy of several chemotherapeutic agents including docetaxel, doxorubicin, or gemcitabine, presumably due to a reduction of the IFP in tumors(22-24).

Here we present the combination of the retargeting RGDK modification of the fiber and the expression of hyaluronidase in a novel oncolytic adenovirus, named VCN-01. This virus shows selective and potent replication in tumor cells in vitro, similar to that of the oncolytic adenoviruses containing the same modifications separately. In vivo, the increased blood persistence associated to the RGDK mutation combined with the hyaluronic acid (HA) degradation driven by hyaluronidase resulted in an improved antitumor efficacy of VCN-01 without increasing the toxicity of the parental virus. Overall, the observed results support the ongoing clinical development of the oncolytic adenovirus VCN-01.
MATERIALS AND METHODS

Cell lines. Human embryonic kidney 293, A549 lung carcinoma, Skmel-28 melanoma, BxPC3, Rwp1 and MiaPaCa-2 pancreatic carcinomas and SCC-25 and SCC-29 head and neck tumor cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). NP-9, NP-18, NP-31, and NP-29 pancreatic tumor cell lines were established in our laboratory(25, 26). HP-1 Syrian hamster pancreatic tumor cell line was obtained from M. Yamamoto (Minneapolis, MN, USA) with MA Hollingsworth (Nebraska, NE, USA) permission. All were maintained with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS, Invitrogen Carlsbad, CA, USA) and penicillin-streptomycin (PS, Gibco-BRL, Barcelona, Spain) at 37ºC, 5%CO₂. All cell lines were routinely tested for mycoplasma contamination by PCR, microbial presence by microscopic observation and antibiotic deprivation and morphology by microscopic observation. Pancreatic islets were kindly provided by Dr Montanya of the Endocrinology Service of the Hospital de Bellvitge, Barcelona, Spain.

Recombinant adenoviruses. Human adenovirus serotype 5 (Adwt) was obtained from ATCC, and AdwtRGDK, AdTLRGDK, ICOVIR-15K, and ICOVIR-17 have been previously described(11, 21, 27). All viruses were propagated in A549 cells. VCN-01 was created by replacing the fiber containing the RGD motif in the HI loop for the RGDK fiber in the ICOVIR-17 genome. To achieve this, an EcoRI digestion fragment of the pBSattKKT plasmid15(11) containing the RGDK fiber was recombined in Saccharomyces cerevisiae YPH857 with the pICOVIR17CAL plasmid (CAU sequence, which includes the yeast autonomous replication elements and a selectable marker for uracil, was replaced by CAL, analogous to CAU but with a selectable marker for leucine instead of uracil in pICOVIR17CAU plasmid (21)) partially digested with Ndel. VCN-01 was obtained by transfection into HEK293 cells of the large PacI fragment of
pICOVIR17RGDKCAL, amplified in A549 cells and purified on CsCl gradient according to standard techniques.

**Assay for hyaluronidase activity.** A549 cells were infected with 20TU/cell. 4h post-infection (pi), cells were washed with PBS and fresh media was added. 72hpi the supernatant (SN) was collected and concentrated with Amicon Ultra centrifugal filters (Millipore, Billerica, MA). SN samples were mixed with a HA (Sigma, St Louis, MO) solution in phosphate buffer (pH 5.35) and were incubated overnight at 37°C. Samples were precipitated with 5 volumes of a solution containing 24mM sodium acetate, 79mM acetic acid and 0.1% of bovine albumin (pH 3.75) and the absorbance at 600nm was read. Units of activity were determined according to a standard curve of hyaluronidase activity, generated from a solution of bovine testicular hyaluronidase (Sigma, St Louis, MO)(28).

**Viral production assays.** Cells were infected with 30TU/cell (Skmel-28 and NP-18) or 20TU/cell (NP-9 and HP-1) of each virus to allow 80-100% infectivity. 4hpi cells were washed 3 times with PBS and incubated with fresh medium. At indicated time points, cells and medium (CE) were harvested and subjected to 3 rounds of freeze-thaw lysis. Viral titers of CE were determined in triplicate according to an anti-hexon staining-based method in HEK293 cells(29).

**In vitro cytotoxicity assays.** 40000 Skmel-28, NP-18 or NP-31, 20000 NP-29 or BxPC-3, 15000 NP-9, Rwp-1, MiaPaCa-2, SCC-29 or SCC-25 or 10000 HP-1 cells/well were seeded in 96-well plates in DMEM 5%FBS. Cells were infected per triplicate with serial dilutions starting with 400TU/cell for SCC-25, 300TU/cell for Rwp-1, MiaPaCa-2 and SCC-29 or 200TU/cell for Skmel-28, NP-18, NP-9, NP-29, NP-31, BxPC-3 and HP-1. Quiescent primary human fibroblast cultures maintained in conditions of over-confluence and serum deprivation were infected per
triplicate with serial 1/3 dilutions of VCN-01 or Adwt-RGDK starting at 0.1TU/cell. At day 5-6pi for tumor cells and day 10 for fibroblasts, plates were washed with PBS and stained for total protein content (bicinchoninic acid assay; Pierce Biotechnology, Rockford, IL, USA). Absorbance was quantified and the TU/cell required to produce 50% inhibition (IC₅₀ value) was estimated from dose-response curves by standard nonlinear regression (GraFit; Erithacus Software, Horley, UK), using an adapted Hill equation.

Replication selectivity in pancreatic islets. Isolated islets in 100µL of CMRL1066 media supplemented with 0.5% of albumin were infected in triplicate with AdTL-RGDK (non-replicative vector), Adwt-RGDK (capsid-modified wild-type virus) or VCN-01 at 200TU/cell (assuming 1000 cells/islet). Infection was performed in suspension and with constant agitation during 2h at 37ºC. Islets were washed 3 times with PBS and seeded in 96-well plates with fresh medium. After washing, a small fraction of the SN was collected and the input virus was titrated by anti-hexon staining (background level). At day 6pi, islets and medium were harvested and freeze-thawed 3 times to obtain the cell extract and the viral titer of each sample was determined.

In vivo studies. Most of the animal studies were performed at the IDIBELL facility (AAALAC unit 1155) and approved by the IDIBELL’s Ethical Committee for Animal Experimentation. Toxicity and biodistribution studies in Syrian hamsters were performed in TNO Triskelion facilities under Good Laboratory Practice (GLP) conditions.

In vivo toxicity study in mice. Vehicle (PBS) or 5x10¹⁰ vp of ICOVIR-17 or VCN-01 were injected intravenously into the tail vein in 6-week-old immunocompetent Balb/C male mice in a finale volume of 200µL (n=1-6). Animals were examined daily for clinical signs of toxicity. At
different time points after the administration of the virus, blood aliquots were collected via tail vein and platelets were counted. At day 7, 12 or 28 post-injection, mice were killed and blood and serum samples were collected by intracardiac puncture. Clinical biochemical and hematological determinations were performed by the Clinical Biochemistry and Hematological Services of the Veterinary Faculty at the Autonomous University of Barcelona. Concentration of several cytokines in sera samples was evaluated at different time points using the Luminex xMAP® technology platform.

In vivo biodistribution study in mice. NP-18 tumors were established by the subcutaneous injection of $1 \times 10^7$ cells into the flanks of 6-week-old female Balb/C nu/nu mice. When tumors reached an average volume of 300-400 mm$^3$, animals were randomized into different treatment groups and treated with a single intravenous injection of vehicle (PBS) or $5 \times 10^{10}$ vp of VCN-01 in a total volume of 150 µL via tail vein. At days 2, 7 and 28 post administration mice were sacrificed and organs were harvested and frozen. Samples were mechanically homogenized and total DNA was extracted according to QIAamp DNA Mini Kit (QIAGEN, Valencia, CA). Genome copy levels were quantified by real-time PCR-based method using VCN-01-specific oligonucleotides (forward primer: 5'-ACATTGCCCAAGAATAAAGACG-3', reverse primer: 5'-TGGAATCAGAAGGAAGGTGA-3').

GLP toxicity and biodistribution in vivo studies in hamster. 5-week-old female Syrian golden hamsters (Mesocricetus auratus) were injected systemically with $2.5 \times 10^{11}$ vp or $4 \times 10^{11}$ vp of VCN-01 or vehicle (Tris Buffer) via cannulation of the jugular vein in a total volume of 270 µL ($n=6$). Animals were examined daily for clinical signs of toxicity and at days 2, 8 and 28 post administration subgroups of hamsters were killed and whole blood and serum samples were collected. Clinical biochemistry of transaminase levels and hematological determinations...
were performed. For biodistribution analysis, organs from animals treated with the higher dose of VCN-01, $4 \times 10^{11}$ vp (n=5), were collected and frozen. Samples were processed and analyzed as reported in the previous section.

**Antitumor activity in mice and hamsters in vivo.** To assess systemic efficacy, subcutaneous Skmel-28 or NP-18 tumors were established by the injection of $9 \times 10^6$ or $5 \times 10^6$ cells respectively into the flanks of 6-week-old male Balb/C nu/nu mice. Once tumors reached the desired mean volume ($100 \text{mm}^3$ for Skmel-28 and $180 \text{mm}^3$ for NP-18), mice were randomized (n=10-16 tumors/group) and treated with a single intravenous injection of vehicle (PBS) or $4.5 \times 10^{10}$ vp of ICOVIR-17 or VCN-01 in a total volume of $200 \mu L$ via tail vein.

To evaluate efficacy after intratumoral administration, Skmel-28, NP-18 or NP-9 tumors were established by the injection of $9 \times 10^6$, $2 \times 10^6$ or $5 \times 10^6$ cells respectively into the flanks of 6-week-old male Balb/C nu/nu mice. When the tumors reached an appropriate mean volume ($150 \text{mm}^3$ for Skmel-28 and NP-18 and $80 \text{mm}^3$ for NP-9) received a single intratumoral injection of PBS or $2 \times 10^6$ vp of VCN-01 in the case of Skmel-28 and NP-9 and $4 \times 10^6$ vp in NP-18 tumors in a final volume of $25 \mu L$. HP-1 tumors were established by injection of $5 \times 10^5$ cells into the flanks of 6-week-old female immunocompetent Syrian hamsters. Once tumors reached the desired mean volume ($220 \text{mm}^3$), were injected with vehicle or $2.5 \times 10^{10}$ vp of VCN-01 (n=10 tumors/group). In all animal experiments, tumor progression and morbidity status were monitored three times weekly. Tumor volume was defined by the equation $V(\text{mm}^3) = \pi/6 \times W^2 \times L$, where W and L are width and length of the tumor, respectively. The percentage of growth was calculated as $((V-V_0)/V_0 \times 100)$, where $V_0$ is the tumor volume on day 0.

**Tumor histochemistry.** Hyaluronic acid and adenovirus staining with anti-E1A antibody staining was performed as previously described.(21, 30). Masson trichromic staining was
performed using the Accustain Trichrome Stain kit (Sigma Aldrich) according to the manufacturer's indications.

**Statistical analysis.** Two-tailed Student's t-test was used to evaluate the statistical significance between groups except for hamster toxicity analysis, in which Kruskal-Wallis test was used, and for Kaplan-Meier survival curves, where log-rank test was performed.
RESULTS

In vitro characterization of VCN-01. Parental viruses ICOVIR-15K, which has the KKTK to RGDK fiber shaft replacement, and ICOVIR-17, which expresses hyaluronidase, have been described before and were used as controls for the in vitro characterization assays (12, 21). As VCN-01, they are based in ICOVIR-15, a virus with E1a mutated in the pRB-binding site and under the control of minimal E2F binding sites to allow for selective replication in a broad range of tumor cells with pRB pathway alterations (31). Note that we apply the “ICOVIR” nomenclature to our viruses with these E1a modifications to achieve tumor-selective replication.

An enzymatic activity assay performed using supernatants of infected cells confirmed hyaluronidase expression by VCN-01 at similar levels than ICOVIR-17 (Figure 1a). To measure the effect of RGDK and hyaluronidase combination on virus fitness, the yield of VCN-01 was compared to ICOVIR-15K and ICOVIR-17 in different tumor cell lines. Despite a minor delay on replication in two cell lines at 24 hours post-infection, VCN-01 production was similar to control viruses at later time points (Figure 1b). Cytotoxicity in a panel of tumor cell lines was also evaluated as an index of virus replication potency. All viruses induced a similar cytotoxic profile with relative VCN-01 IC₅₀ values ranging from half to three-fold compared to the parental viruses (Table 1).

VCN-01 replication was studied in two human primary normal cell models to check if replication was tumor-selective. Primary human fibroblasts were made quiescent by over-confluence and serum deprivation, and the ability of VCN-01 and RGDK wild-type adenovirus (AdwtRGDK) to kill them was assessed by infecting the cells with serial viral dilutions to determine the viability percentage at day 10 post-infection. VCN-01 demonstrated to be less cytotoxic in this model than the non-selective AdwtRGDK (Figure 1c). Selectivity was also evaluated in human pancreatic islets in vitro, as pancreatic cancer patients are candidates for
treatment. Same viral progeny levels were detected at day 6 post-infection in the samples infected with VCN-01 and the non-replicative negative control (AdTLRGDK), whereas wild-type adenovirus (AdwtRGDK) replicated in normal human pancreatic islets (Figure 1d).

**In vivo toxicity upon VCN-01 systemic administration in immunocompetent mice.** Balb/C immunocompetent mice were injected with vehicle or $5 \times 10^{10}$ viral particles (vp) of ICOVIR-17 (selected for comparative studies as it is the most efficacious parental virus) or VCN-01 via tail vein to assess toxicity after intravenous administration. Weight loss, liver enzymes (aspartate aminotransferase, AST, and alanine aminotransferase, ALT), hematological parameters and viremia were determined at different time points. Wild type adenovirus 5 (Adwt) was included as a control, but given the high toxicity associated with its administration, animals were sacrificed at day 3. VCN-01 caused a similar and reversible body weight loss profile to that of ICOVIR-17, reaching the maximum loss (9 %) at day 7 post-administration (Figure 2a). Other toxicological events observed at day 7 were moderate nonsignificant increases in AST (2.9-fold for ICOVIR-17 and 2.4-fold for VCN-01) and ALT levels (12-fold for ICOVIR-17 and 9-fold for VCN-01) (Figure 2b), thrombocytopenia (Figure 2c), and increased monocytes and neutrophils counts (Figure 2d), but at days 12 and 28 these parameters were normal.

A higher viremia was detected in VCN-01-treated animals compared with ICOVIR-17 (17.5, 8.7 and 4.1-fold at 5, 15, and 60 minutes, respectively). Moreover, half-life of VCN-01 was significantly higher (3.15 versus 2.11 minutes for ICOVIR-17) (Supplementary Figure S1). To evaluate the innate immune response elicited by the systemic exposure to VCN-01, eight cytokines were measured at different time points. Virus administration induced a rapid statistically significant rise in blood levels of IFN-γ, IP-10, IL-6, and TNF-α that were normalized by day 10 except for IP-10, which remained significantly elevated (Figure 2e).
In vivo toxicity upon VCN-01 systemic administration in Syrian hamster. A GLP toxicity study was performed in Syrian hamsters (Mesocricetus auratus), considered more permissive to human adenovirus replication than mice (32). Hamsters were injected intravenously with vehicle, $2.5 \times 10^{11}$ (low dose) or $4 \times 10^{11}$ (high dose) vp of VCN-01. At days 2, 8, and 28 post-administration, subgroups of animals were sacrificed and body weight loss, clinical chemistry of the blood, and hematology were studied. Several parameters were altered at day 2 in the animals treated with the high dose of VCN-01. Most notable were the 7% body weight loss (Figure 3a), elevation in transaminase levels (65.1-fold for AST and 23.8-fold for ALT) (Figure 3b), alkaline phosphatase (ALP) levels (Supplementary Figure S2a), thrombocytopenia (Figure 3c) accompanied with an increased prothrombin time (PTT) (Supplementary Figure 2d), and higher monocyte and neutrophil counts (Figure 3d), although at days 8 and 28 these parameters were normalized. These changes were dose-dependent and associated to histopathological changes in the liver, characterized by necrosis, nuclear inclusion bodies, and hemorrhages, findings that had subsided in animals sacrificed 8 or 28 days post injection (data not shown). Any alterations were detected in creatinine or urea levels, indicating no renal toxicity (Supplementary Figures S2b and S2c).

Biodistribution of VCN-01 upon intravenous administration in vivo. Mice carrying NP-18 human xenografts were treated with a single intravenous dose of $5 \times 10^{10}$ vp. At days 2, 7, and 28 post-administration, animals were sacrificed and viral genomes were quantified by quantitative real-time PCR analysis in target tissue (tumors) and in other relevant non-target organs (liver and spleen). Viral genomes were detected in the three tissues at the earliest time point (day 2), with most of the virus present in the liver (Figure 4a). Importantly, at days 7 and 28, this organ was negative for VCN-01 genomes. The clearance of a lower amount of virus
found in the spleen seemed delayed compared to the liver. In contrast, VCN-01 genomes increased over time in tumors, indicating replication.

Since hamster cells are semi-permissive to adenovirus replication, a GLP biodistribution study was carried out in individuals without tumors as complementary to safety study presented above, in animals receiving the high intravenous dose of $4 \times 10^{11} \text{vp/hamster}$. Viral genomes presence was analyzed in different organs at days 2, 8, and 28. At day 2, viral DNA was found in all tissues tested, with highest levels in liver (Figure 4b, different panels), although only residual amounts were detected at days 8 and 28. As mentioned above, the presence of virus at this late time points was not associated to morphology changes. Specific evaluation of ovaries by fluorescent in situ hybridization (FISH) revealed no presence of virus DNA in germinal cells (data not shown).

**Antitumor activity of VCN-01 upon systemic administration.** VCN-01 efficacy after systemic administration was compared with that of its non-RGDK counterpart, ICOVIR-17, in order to assess if RGDK modification provides an improvement in this context, as previously reported with ICOVIR-15(12). Mice carrying Skmel-28 or NP-18 tumors were injected intravenously with vehicle or $4.5 \times 10^{10} \text{vp}$ of ICOVIR-17 or VCN-01 and tumor growth was monitored. In Skmel-28-bearing mice, antitumor efficacy was maintained throughout the study (for up to 83 days) in VCN-01-treated animals, whereas after a similar curve of initial antitumor activity, a relapse was observed by day 53 post-treatment in ICOVIR-17 group (Figure 5a, left panel). Statistical differences in the percentage of tumor growth between both groups were observed from day 53 post-treatment until the end of the study ($p=0.00027$), when tumor size was 2.4-fold larger in the ICOVIR-17-treated animals than in the VCN-01-injected ones. Moreover, 50% of VCN-01-treated tumors were regressing at this final time point. A similar result was observed in mice bearing NP-18 tumors, in which a greater control of the tumor
growth was observed in the animals treated with VCN-01 than with ICOVIR-17 (Figure 5a, right panel). These differences were significant from day 30 to day 49 post-administration (p=0.0132), when the study ended.

**Antitumor activity of VCN-01 upon intratumoral administration.** VCN-01 efficacy after intratumoral administration was also studied given the intended clinical application of this administration route. Mice carrying Skmel-28, NP-9 or NP-18 human xenografts were treated with a single intratumoral injection of vehicle or $2 \times 10^8$ vp of VCN-01 for Skmel-28 and NP-9. For NP-18, $4 \times 10^9$ vp were injected as it is a faster-growing model compared to the other two. To reduce animal number, no further comparison with ICOVIR-17 was considered. Significant reduction in tumor growth could be seen in all three models and, at the end of the studies, the mean tumor size of VCN-01-treated groups was 4.2- and 1.6-fold smaller compared to non-treated tumors in Skmel-28 and NP-9 models, respectively. In the case of NP-18, mock-treated animals had to be killed at day 18 post-administration due to large tumors, when mean tumor size of VCN-01-treated tumors was 3.6-fold smaller compared to the control (Figure 5b, different panels).

Since mice are not permissive to Ad5 replication, a hamster model was included to evaluate the antitumor efficacy in the presence of immune system. HP-1 tumors were treated with a single intratumoral injection of vehicle or $2.5 \times 10^{10}$ vp of VCN-01. Virus treatment significantly reduced tumor growth from day 9 post-administration until the end of the study by day 28, when tumor volume was 2.1-fold smaller in VCN-01-treated animals (Figure 5b). VCN-01 was able to significantly increase the survival of the animals in all tumor models tested in mice and hamsters (Supplementary Figure S3).
Changes induced by VCN-01 in the extracellular matrix of tumors. In order to characterize the activity of VCN-01 and the histological changes produced in the ECM of the tumors at late time points, we performed studies in NP-18 tumors on day 78 after VCN-01 intratumoral treatment. The intratumoral replication of VCN-01 was demonstrated by quantification of viral genomes by real-time PCR (Figure 5c, upper panel) and E1A immunohistochemistry in tumor sections (Figure 5c, lower panel). Histochemical analysis of hyaluronic acid (HA) showed that, whereas in vehicle-treated tumors HA was extensively and homogenously distributed among tumor mass, tumors treated with VCN-01 showed a dramatic decrease in the intratumoral HA content (Figure 5d, upper panel). Additionally, Masson trichromic staining was used to detect connective tissue and collagen fibers. Similar to HA, collagen was homogeneously distributed in vehicle-injected tumors. In contrast, those treated with VCN-01 displayed collagen structures forming bundles that surrounded infected zones (Figure 5d, lower panel).
DISCUSSION

Oncolytic viruses can potentially eliminate tumor cells directly by lysis or indirectly by immune responses. Antitumor immunity is so appealing that most viruses in clinical trials are armed with immunostimulatory genes(1, 2). Nevertheless, improving oncolytic traits should not be underestimated since large tumors may need extensive tumor debulking by viral infection and replication in order to be rejected by Cytotoxic T lymphocytes (CTLs). Therefore, the arrival of the virus to the tumors and intratumoral spread are important factors to be improved(3).

In the present work, we have combined two different modifications previously described by our group addressing both of these issues in a single oncolytic adenovirus, VCN-01: the KKTK to RGDK replacement to improve the half-life in blood of the virus after systemic administration(11, 12), and the expression of hyaluronidase to enhance the intratumoral spread(21). We hypothesized that this combination in a highly active oncolytic adenovirus of broad applicability as our previously described ICOVIR-15(31) would generate an interesting candidate for clinical development. In this context, it is worthy to highlight that DNX-2401 (formerly Ad-D24RGD), a parental virus to ICOVIR-15, has received fast-track designation from the FDA after promising results in a Phase I trial in glioblastoma(33).

For safety in clinical development, impaired VCN-01 replication was demonstrated in two different non-tumor models in vitro, and different safety studies were conducted in two rodent species in vivo: mouse and hamster. Mice are poorly permissive to Ad5 replication(34); nevertheless, this animal model allow the study of toxicity associated to the viral capsid, the expression of early adenoviral genes such as E1A, and the innate immune responses mainly triggered by the capsid(35) since the cytokine pattern induced by systemic administration of adenoviruses in mice is similar to humans(36, 37). Syrian hamsters are considered semi-permissive to human adenovirus replication and have been used to evaluate the toxicological
events related to the expression of late adenoviral genes(32). Despite the differential permissiveness, similar toxicity profile was observed in both species, suggesting its independency on viral replication. Toxicity was dose-dependent and mainly due to direct infection of liver cells and the consequent expression of early viral proteins. The acute inflammatory cytokine-mediated immunity induced within the first 72 hours after VCN-01 administration also contributed to liver pathology. Although this initial cytokine induction could have some implications in antitumor activity, this seems unlikely as non-replicative vectors, that induce cytokines(38), do not show antitumor activity(39). Importantly, since VCN-01 replication is impaired in normal tissues, toxicity was transient and disappeared once the levels of viral proteins decreased in initially infected cells(40). Accordingly, adverse events observed in hamsters were normalized by day 8. In summary, VCN-01 toxicology data obtained in the preclinical studies is similar to the previously described for ICOVIR-15, ICOVIR-15K and, ICOVIR-17(12, 21) and detected alterations match with the most common toxicity events reported after systemic administration of adenoviruses in clinical trials(35-37, 41). Biodistribution studies were consistent with the toxicology pattern, with highest levels of viral genomes in the liver at early time points, in agreement with previous reports(32, 42-45). However, at later time points only residual traces of viral DNA were observed in liver as well as in other non-target organs. These results are especially relevant in hamster model, in which replication of the virus occurs at a certain level. In contrast, the increase over time in viral genome levels in tumor samples of the human xenograft model in mice indicated active and selective replication of VCN-01.

We observed that compared to a virus which has the RGD motif inserted in the HI loop, the RGDK fiber shaft mutation does not increase toxicity upon intravenous administration. Nevertheless, this mutation significantly improved the antitumor activity of ICOVIR-17 after a single intravenous administration in two different tumor models in vivo (Skmel-28 and NP-18).
This fact is very relevant since ICOVIR-17 was already the result of a series of steps to optimize oncolytic potency(21). Efficacy via intratumoral administration was also evaluated as this route has been commonly used to treat tumor types such as pancreatic adenocarcinoma, glioblastoma, and head and neck adenocarcinoma. A single intratumoral injection of VCN-01 significantly reduced tumor growth and increased survival when compared to control group in all tumor models tested. HP-1 model was of particular interest as hamsters have been proposed as a good model to study oncolytic adenoviruses in an immunocompetent environment. However, in our hands, the replication permissiveness is much lower than in human cells. In addition, hamsters develop an anti-adenoviral immune response which causes viral clearance and prevents subsequent infection of tumor cells even after intratumoral administration. Thomas and colleagues suggested to immunosuppress hamsters with drugs like cyclophosphamide (CP) as a way to increase antitumor efficacy, allowing sustained viral replication and oncolysis(46). Certainly, efficacy studies in Ad5 seropositive models or upon repeated administration in immunocompetent animals and in combination with immunomodulation or chemotherapy will be needed to better understand the potential and limitations of VCN-01.

Arming oncolytic viruses with ECM degrading enzymes is a commonly exploited strategy to enhance viral penetration of solid tumors(14). Different modulators of ECM such as decorin, relaxin, metalloprotease 9, or chondroitinase ABC have been used to increase viral spread and antitumor efficacy in different tumor types taking into account specific ECM compositions(16-20). High levels of HA are present in almost 87% of pancreatic adenocarcinomas(22, 47), and hyaluronidase expression by VCN-01 can provide a particular advantage to treat this tumor type. As it is shown in figure 5d, hyaluronidase activity causes a dramatic decrease in HA content of treated tumors that may facilitate intratumoral virus spread. Furthermore, early clinical trials have demonstrated that hyaluronidase enhances chemotherapy efficacy in cancer patients (48). Preclinical studies by Jacobetz and coworkers have attributed the beneficial effect of
hyaluronidase to a lower interstitial pressure in HA-depleted tumors, leading to blood vessel decompression and increasing the vascular permeability, thereby favoring the penetration of drugs to the tumor core(23). These studies strongly suggest the possibility of combining VCN-01 with chemotherapies such as gemcitabine, which is the current standard-of-care treatment in pancreatic ductal adenocarcinoma. Additionally, hyaluronidase administration expressed from an oncolytic virus provides some advantages compared to recombinant enzymes. VCN-01 restricts transgene expression to tumor sites and, consequently, may limit systemic side effects. In fact, a recent phase II clinical trial testing PEGPH20 in combination with chemotherapy (NCT01839487) was transiently halted because of associated toxicity. Moreover, in contrast to recombinant enzymes which have a relatively short half-life and may require readministrations, sustained delivery of hyaluronidase could be achieved with a replicating virus.

Despite the notable improvement of antitumor activity obtained with VCN-01, complete tumor regression were rare. Masson staining of tumors suggests that collagen bundles surround the infected areas of the tumors, impairing the spread of the virus. Besides ECM and malignant cells, solid tumors contain stromal cells such as cancer-associated fibroblasts (CAFs), endothelial, and inflammatory cells which physically limit viral spread. In this sense, it would be interesting to combine the enzymatic degradation of the ECM by hyaluronidase with a strategy directed to eliminate stromal cells. Our group recently described that the truncation of the i-leader adenoviral protein enhanced the release and cytotoxicity of the virus in CAFs in vitro and increased its antitumor activity in vivo(49, 50).

In conclusion, VCN-01 addresses two of the main limitations in the treatment of cancer with oncolytic adenoviruses: tumor targeting and spread across the ECM barrier. Altogether, the present study supports VCN-01 as a potential candidate for clinical development. Currently, two Phase I clinical trials are ongoing; one in pancreatic cancer by endoscopic ultrasound-guided
intratumoral injection and another one targeting different tumor types by intravenous administration (NCT02045602 and NCT02045589).
ACKNOWLEDGEMENTS

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REFERENCES


Table 1. Comparative cytotoxicity *in vitro* of VCN-01, ICOVIR-15K, and ICOVIR-17 in different tumor cell lines.

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Cell line</th>
<th>IC₅₀ (TU/cell)</th>
<th>Cytotoxicity increase vs ICOVIR-15K</th>
<th>Cytotoxicity increase vs ICOVIR-17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICOVIR-15K</td>
<td>ICOVIR-17</td>
<td>VCN-01</td>
<td></td>
</tr>
<tr>
<td>Human melanoma</td>
<td>Skmel-28</td>
<td>0.36 ± 0.05</td>
<td>0.25 ± 0.02</td>
<td>0.54 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>NP-18</td>
<td>0.12 ± 0.01</td>
<td>0.15 ± 0.02</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>NP-9</td>
<td>2.94 ± 0.74</td>
<td>1.34 ± 0.22</td>
<td>2.44 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>NP-29</td>
<td>3.31 ± 0.63</td>
<td>1.90 ± 0.22</td>
<td>3.57 ± 0.50</td>
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<tr>
<td>Human pancreatic adenocarcinoma</td>
<td>NP-31</td>
<td>5.58 ± 1.13</td>
<td>2.11 ± 0.61</td>
<td>3.47 ± 0.83</td>
</tr>
<tr>
<td></td>
<td>BxPC-3</td>
<td>1.31 ± 0.36</td>
<td>1.61 ± 0.19</td>
<td>1.43 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>Rwp-1</td>
<td>0.07 ± 0.003</td>
<td>0.076 ± 0.007</td>
<td>0.053 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>MiaPaca2</td>
<td>0.091 ± 0.007</td>
<td>0.076 ± 0.003</td>
<td>0.074 ± 0.007</td>
</tr>
<tr>
<td>Hamster pancreatic adenocarcinoma</td>
<td>HP-1</td>
<td>1.03 ± 0.12</td>
<td>1.28 ± 0.12</td>
<td>0.70 ± 0.06</td>
</tr>
<tr>
<td>Human head and neck adenocarcinoma</td>
<td>SCC-29</td>
<td>2.47 ± 0.18</td>
<td>1.64 ± 0.24</td>
<td>0.78 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>SCC-25</td>
<td>27.23 ± 4.13</td>
<td>35.44 ± 3.20</td>
<td>29.10 ± 7.28</td>
</tr>
</tbody>
</table>

Cells were infected with the indicated viruses at doses ranging from 400 to 0.0002 transduction units (TU) per cell. IC₅₀ values (TU per cell required to cause a reduction of 50% in cell culture viability, n=3) ±SD at day 5-10 after infection and VCN-01 cytotoxicity increase are shown.
FIGURE LEGENDS

Figure 1. In vitro characterization of VCN-01. (a) Hyaluronidase expression levels of VCN-01. Hyaluronidase activity was evaluated in concentrated supernatants of A549 cells 72 hours after the infection with ICOVIR-15K, ICOVIR-17 or VCN-01 by digesting hyaluronic acid (HA) samples with these supernatants. Mean values +SD are plotted. *, significant (p<0.05) by two-tailed unpaired Student’s t-test compared to ICOVIR-15K group. (b) Viral production of VCN-01 in tumor cells. Different tumor cell lines were infected at high multiplicity of infection (MOI) with ICOVIR-15K, ICOVIR-17 or VCN-01. At indicated time points, cell extracts were harvested and titrated by an anti-hexon staining-based method. Mean values +SD are shown. *, VCN-01 significant (p<0.05) by two-tailed unpaired Student’s t-test compared to ICOVIR-15K group; #, VCN-01 significant (p<0.05) by two-tailed unpaired Student’s t-test compared to ICOVIR-17 group. (c) Replication selectivity of VCN-01 in vitro. Human fibroblasts were infected with serial dilutions of VCN-01 and AdwtRGDK (wild type non-selective adenovirus) and cell viability was determined at day 10 post-infection. Mean values of percentage of cell mortality +SD are plotted (left panel). (d) Human primary pancreatic islets were infected with AdTLRGDK (non-replicative virus), AdwtRGDK and VCN-01. 6 days post infection islets and culture supernatant were collected and total virus content was determined according to an anti-hexon staining-based method. Mean+SD is plotted (right panel). *, VCN-01 significant (p<0.05) by two-tailed unpaired Student’s t-test compared to AdwtRGDK. IU, International units; TU, transduction units.

Figure 2. Toxicity profile after systemic administration of VCN-01 in immunocompetent mice. The average values for (a) body weight variation, (b) serum transaminase levels, (c) platelets concentration and (d) blood cell counts in Balb/C peripheral blood at indicated time
points after intravenous administration of 5x10^{10} viral particles per mouse of Adwt (wild-type Ad5), ICOVIR-17 or VCN-01 are shown. Adwt-injected mice were sacrificed at day 3 due to lethal toxicity. Mean values +SD are depicted. (e) Average concentration values of IFN-γ, IL-2, IL-6, IL-10, IP-10, TNF-α, GM-CSF, and IL-1β cytokines at indicated time points assessed by Luminex xMAP® technology platform. Mean +SEM is plotted (n=3-6). *, VCN-01 significant (p<0.05) by two-tailed unpaired Student’s t-test, compared to vehicle group. #, VCN-01 significant (p<0.05) compared to Adwt. δ, VCN-01 significant (p<0.05) compared to ICOVIR-17 group. Normal AST and ALT values in male Balb/C mice are 135 IU/L (95% interval 55-352) and 60 IU/L (95% interval 41-131), respectively. ALT, alanine aminotransferase; AST, aspartate aminotransferase; IU, International units.

**Figure 3.** Toxicity profile after systemic administration of VCN-01 in immunocompetent Syrian hamster. The average values for (a) body weight variation, (b) serum transaminase levels, (c) platelets concentration and (d) blood cell counts in hamsters peripheral blood at indicated time points after intravenous administration of 2.5x10^{11} or 4x10^{11} viral particles of VCN-01 are shown. Mean values +SD are depicted. *, VCN-01 high dose significant (p<0.05) by Kruskall-Wallis test, compared to vehicle group. #, VCN-01 high dose significant (p<0.05) compared to VCN-01 low dose. Normal AST and ALT values in female Syrian hamster are 32 ± 13.2 IU/L and 29 ± 10.4 IU/L, respectively. ALT, alanine aminotransferase; AST, aspartate aminotransferase; IU, International units.

**Figure 4.** Biodistribution profile of VCN-01 after intravenous administration. (a) Balb/C nude mice carrying NP-18 (pancreatic adenocarcinoma) human xenograft tumors or (b) immunocompetent Syrian hamsters were treated intravenously with VCN-01 at a dose level of 5x10^{10} and 4x10^{11} viral particles respectively. Animals were sacrificed at indicated time points
and different organs were harvested in each case. Samples were mechanically homogenized, total DNA was extracted and VCN-01 genome copy levels were quantified by real-time PCR-based method. VCN-01 genome copies in 100 nanograms of total DNA for vehicle group was set as 1 (control) and data are expressed as number of VCN-01 genome copies fold over control ±SEM (n=4-18 samples/group). *, VCN-01 significant (p<0.05) by two-tailed unpaired Student’s t-test, compared to vehicle group.

**Figure 5.** Antitumor activity of VCN-01. (a) Antitumor efficacy after intravenous administration. Nude mice bearing subcutaneous xenografts of Skmel-28 (melanoma) or NP-18 (pancreatic adenocarcinoma) tumors were injected intravenously with vehicle or 4.5x10^10 viral particles per mouse of ICOVIR-17 or VCN-01. Mean percentage of tumor growth value +SEM is plotted (n=10 tumors/group). *, VCN-01 significant p<0.05 by two-tailed unpaired Student’s t-test compared with mice injected with vehicle. #, VCN-01 significant p<0.05 by two-tailed unpaired Student’s t-test compared with mice injected with ICOVIR-17 (from day 53 to day 83 in Skmel-28 model and from day 30 to day 49 in NP-18 model). (b) Antitumor efficacy after intratumoral administration. Skmel-28 (melanoma), NP-18 or NP-9 (pancreatic carcinoma) xenograft tumors in nude mice were treated intratumorally with a single injection of vehicle or VCN-01 at a dose of 2x10^9 (Skmel-28 and NP-9) or 4x10^9 (NP-18) viral particles per tumor. Syrian hamster bearing HP-1 (hamster pancreatic carcinoma) subcutaneous tumors were treated with one intratumoral injection of vehicle or 2x10^11 viral particles per tumor of VCN-01. Mean percentage of tumor growth +SEM is plotted (n=8-14 tumors/group). *, significant p<0.05 by two-tailed unpaired Student’s t-test compared to control group. Presence of VCN-01 in NP-18 (pancreatic adenocarcinoma) tumors 78 days after treatment with a single intratumoral injection of 4x10^9 viral particles was assessed by viral genomes quantification by real-time PCR method in DNA extracted from homogenized tumors (c, upper panel, mean+SD is plotted) and by
immunohistochemical staining of the early viral protein E1A (c, lower panel; original magnification x400). Hyaluronic acid (d, upper panel; original magnification x100) and Masson trichromic staining (d, lower panel, original magnification x100) were also performed in deparaffinized tumor sections. Black dots marks nuclei, red areas reveal keratin and acidic fibers, pink marks cytoplasm and blue stain collagen fibers.*, significant p<0.05 by two-tailed unpaired Student’s t-test compared with mice injected with vehicle.
Figure 1

(a) 

(b) 

Skmel-28

NP-18

NP-9

HP-1

Percentage of cell mortality

Produced viruses (TU/cell)
Figure 2

(a) Percentage of body weight change over time after vehicle, Adwt, ICOVIR-17, and VCN-01 treatments. (b) Serum levels of AST and ALT on Day 7, 12, and 28 for vehicle, ICOVIR-17, and VCN-01. (c) Change in cell count over time after vehicle and VCN-01 treatments. (d) Differentiation of lymphocytes, monocytes, T-cells, and neutrophils on Day 7, 12, and 28 for vehicle, ICOVIR-17, and VCN-01. (e) Expression of IFN-γ, IL-2, IL-6, IL-10, IP-10, TNF-α, GM-CSF, and IL-1β over time for vehicle and VCN-01 treatments.
Figure 4

(a) Fold over control

Liver  Spleen  Tumor

Vehicle  VCN-01 Day 2  VCN-01 Day 7  VCN-01 Day 28

(b) Fold over control

Liver  Spleen  Lung  Kidney

Heart  Pancreas  Brain  Muscle

Blood  Lymph  Ovary

Vehicle  VCN-01 Day 2  VCN-01 Day 8  VCN-01 Day 28
Figure 5

(a) Percent of tumor growth over time for Skmel-28 cells with different treatments. 
(b) Percent of tumor growth for other cell lines with VCN-01 treatment. 
(c) Comparison of genomic DNA content with different treatments. 
(d) Immunohistochemical staining results for different treatments.
Safety and efficacy of VCN-01, an oncolytic adenovirus combining fiber HSG-binding domain replacement with RGD and hyaluronidase expression

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