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Phase I, multicenter, open-label study of intravenous VCN-01 oncolytic adenovirus with or without nab-paclitaxel plus gemcitabine in patients with advanced solid tumors

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ABSTRACT

Background VCN-01 is an oncolytic adenovirus (Ad5 based) designed to replicate in cancer cells with dysfunctional RB1 pathway, express hyaluronidase to enhance virus intratumoral spread and facilitate chemotherapy and immune cells extravasation into the tumor. This phase I clinical trial was aimed to find the maximum tolerated dose/recommended phase II dose (RP2D) and dose-limiting toxicity (DLT) of the intravenous delivery of the replication-competent VCN-01 adenovirus in patients with advanced cancer.

Methods Part I: patients with advanced refractory solid tumors received one single dose of VCN-01. Parts II and III: patients with pancreatic adenocarcinoma received VCN-01 (only in cycle 1) and nab-paclitaxel plus gemcitabine (VCN-concurrent on day 1 in Part II, and 7 days before chemotherapy in Part III). Patients were required to have anti-Ad5 neutralizing antibody (NAbs) titers lower than 1/350 dilution. Pharmacokinetic and pharmacodynamic analyses were performed.

Results 26% of the patients initially screened were excluded based on high NAbs levels. Sixteen and 12 patients were enrolled in Part I and II, respectively: RP2D were 1×10^{13} viral particles (vp)/patient (Part I), and 3.3×10^{12} vp/patient (Part II). Fourteen patients were included in Part III: there were no DLTs and the RP2D was 1×10^{13} vp/patient. Observed DLTs were grade 4 aspartate aminotransferase increase in one patient (Part I, 1×10^{13} vp), grade 4 febrile neutropenia in one patient and grade 5 thrombocytopenia plus enterocolitis in another patient (Part II, 1×10^{13} vp). In patients with pancreatic adenocarcinoma overall response rate were 50% (Part II) and 50% (Part III). VCN-01 viral genomes were detected in tumor tissue in five out of six biopsies (day 8). A second viral plasmatic peak and increased hyaluronidase serum levels suggested replication after intravenous injection in all patients. Increased levels of immune biomarkers (interferon-γ, soluble lymphocyte activation gene-3, interleukin (IL)-6, IL-10) were found after VCN-01 administration.

Conclusions Treatment with VCN-01 is feasible and has an acceptable safety. Encouraging biological and clinical activity was observed when administered in combination with nab-paclitaxel plus gemcitabine to patients with pancreatic adenocarcinoma.

Trial registration number NCT02045602.

INTRODUCTION

The use of replication-competent oncolytic viruses is a promising new option in the treatment of patients with cancer. Their mechanism of action is based on their intrinsic ability for intracellular replication in target tumor cells that leads to oncolytic activity. Regulatory agencies have so far approved two genetically engineered oncolytic viruses for clinical use via intratumoral administration. Oncorine (H101), a type 5 adenovirus approved in 2005 in China for patients with head and neck tumors,[1 2] and T-Vec (talimogene laherparepvec), a type 1 herpes simplex approved in 2015 by the Food and Drug Administration in the USA (and later by the European Medicines Agency and the Therapeutic Goods Administration in Europe and Australia, respectively) for the treatment of advanced melanoma.[3] The switch from intralesional to systemic delivery is challenged by limitations in systemic bioavailability and collateral damages to healthy tissues. The rapid plasmatic clearance of the viruses by liver, spleen, and macrophages[4] limits their

bioavailability[5] due to viral sequestration in the liver with a high risk of hepatotoxicity.

VCN-01 is a new oncolytic replication-competent adenovirus designed to overcome these challenges.[6] VCN-01 derives from ICOVIR-15[7] and ICOVIR-15K,[8] which replicate only in cells with a dysfunctional RB1 pathway. VCN-01 has been genetically engineered with two essential modifications giving advantage over its parental viruses. First, it incorporates an RGKD integrin motif in the fiber shaft, an effective method for liver detargeting and tumor retargeting. In addition, VCN-01 expresses a soluble form of human recombinant hyaluronidase (PH20), which enables the virus to degrade the extracellular matrix, disrupt the tumor stroma, thus enhancing the intratumoral spreading of the virus.[9]

Despite recent advances, the prognosis of patients with pancreatic cancer remains poor, with median survival of less than 1 year with best available therapy. Single-agent gemcitabine has been the standard of care until recently. Nab-paclitaxel plus gemcitabine improved progression-free survival (PFS) and overall survival (OS) compared with gemcitabine alone[10] and became a new standard for first-line treatment. The addition of intravenous VCN-01 to nab-paclitaxel plus gemcitabine to treat these patients could potentially improve efficacy without a substantial increase in toxicity. With this in mind, we designed a phase I clinical trial to evaluate the safety, tolerability, pharmacokinetics (PK), maximum tolerated dose (MTD), recommended phase II dose (RP2D), and early evidence of biological and clinical activity of intravenous VCN-01 alone or associated with nab-paclitaxel plus gemcitabine in patients with ductal pancreatic adenocarcinoma (PDAC) and other solid tumors.

METHODS

Study design, participants, and treatment

Pancreatic cancer was from the very beginning the target of VCN-01. This study was designed as a multicenter, open-label, dose-escalation phase I clinical trial of intravenous VCN-01 alone (Part I) or in combination with nab-paclitaxel plus gemcitabine (Part II and III). online supplemental appendix figure 1 summarizes the study design. Key inclusion criteria were as follows:

Part I: In order to accelerate drug development, Part I was opened to histologically confirmed locally advanced or metastatic, unresectable, solid tumors whose disease has progressed despite standard therapy or for whom no standard treatment exists. All patients included in this part of the trial had received prior systemic therapy for a variety of solid tumors, mostly colorectal cancer.

Parts II and III: Part II and III of the protocol included patients with locally advanced or metastatic, unresectable, pancreatic adenocarcinoma, for whom gemcitabine and nab-paclitaxel was considered an appropriate treatment regimen by treating physicians. Therefore, prior systemic therapy was allowed but not mandatory, and no prespecified limit of prior therapies was defined per protocol. As reported in table 1, 85% of patients with PDAC included had metastatic disease (10 of 12 in Part II, 12 of 14 in Part III) and 77% had received no prior systemic therapy (8 of 12 in Part II, 12 of 14 in Part III).

VCN-01 was supplied as a suspension in 20 mM Tris buffer at pH 8.0, containing 25 mM NaCl and 2.5% glycerol. A total of 50 mL of VCN-01 was diluted to the correct dose level using 0.9% NaCl. In all three parts, VCN-01 was administered by intravenous infusion over 10 min with patient monitoring.

Investigational treatment in Part I was one single dose of VCN-01 on day 1, according to a preplanned dose-escalation schedule (see below and more details in online supplemental appendix figure 2).

Treatment in Part II consisted of one single injection of VCN-01 on day 1 followed by a 30 min intravenous infusion of nab-paclitaxel (125 mg/m^2) and a 30 min intravenous infusion of gemcitabine (1000 mg/m^2). Nab-paclitaxel and gemcitabine were administered again on days 8 and 15 of a 28-day cycle. Subsequent cycles consisted of nab-paclitaxel plus gemcitabine at the same schedule (days 1, 8 and 15) without VCN-01. Length of all cycles was 28 days.

Treatment in Part III consisted of one single injection of VCN-01 on day 1 followed, 7 days later, by nab-paclitaxel (125 mg/m^2) plus gemcitabine (1000 mg/m^2) on days 8, 15, and 22 for a 35-day cycle (delayed schedule). Subsequent cycles consisted of nab-paclitaxel plus gemcitabine at the standard schedule (days 1, 8 and 15) without VCN-01. Length of all cycles was 28 days except for the 35 days of the first one.

In all three parts, key exclusion criteria were active infection or any other serious illness, autoimmune disease or condition requiring chronic immunosuppressive therapy, levels of neutralizing antibodies against adenovirus >1/350 dilution (assessed by cell-based functional assay), viral syndrome diagnosed during the 2 weeks before inclusion, Li-Fraumeni syndrome or previously known retinoblastoma protein pathway germinal deficiency.

Endpoints and assessments

MTD was defined as the highest dose at which ≤1 of 6 patients experienced dose-limiting toxicity (DLT) during the first 28-day treatment period (35-day in Part III), with the next higher dose having at least two of the first six patients experiencing a DLT during cycle 1. In this study, RP2D was defined as the MTD or higher non-toxic dose reached for each study part. Primary endpoints were treatment-emergent adverse events (TEAE), MTD, and RP2D. Antitumor activity was assessed by CT or positron emission tomography-computed tomography (PET-CT) per investigators criteria every 8 weeks according to Response Evaluation Criteria In Solid Tumors (RECIST) V.1.1. Secondary endpoints were PK parameters, presence of VCN-01 in tumor tissue, shedding of VCN-01 (presence of the virus in the blood, urine, feces, and sputum), plasmatic levels of neutralizing anti-VCN-01

Table 1 Baseline clinical and pathological characteristics of patients included in the study

	Part I				Part II		Part III	
	(n=16)				(n=12)		(n=14)	
	I-1	I-2	I-3	I-4	II-1	II-2	III-1	III-2
	1×10^{11} vp	1×10^{12} vp	3.3×10^{12} vp	1×10^{13} vp	3.3×10^{12} vp	1×10^{13}vp	3.3×10^{12} vp	1×10^{13}vp
	(n=3)	(n=4)	(n=3)	(n=6)	(n=6)	(n=6)	(n=8)	(n=6)
Characteristics								
Median age, years (range)	66 (37–77)				60 (35–75)		66 (49–86)	
	67 (60–69)	70 (67–77)	72 (64–75)	54 (37–63)	61 (46–75)	59 (35–73)	64 (49–86)	68 (51–77)
Sex (male/female)	2/1	3/1	2/1	5/1	3/3	4/2	3/5	2/4
ECOG								
0	1	2	1	1	2	2	2	2
1	2	2	2	5	4	4	4	4
Primary disease								
Colorectal—stage IV	3	4	3	5	0	0	0	0
Head and neck	0	0	0	1	0	0	0	0
Pancreas–stage III	0	0	0	0	1	1	2	0
Pancreas–stage IV	0	0	0	0	5	5	6	6
Site of metastatic disease								
Liver (only)	1	2	0	1	4	3	2	4
Lung (only)	0	0	0	0	0	0	0	0
Liver and lung (and other)	1	0	1	5	1	1	1	0
Liver (and other)	0	0	0	0	0	1	1	2
Other	1	2	2	0	0	0	2	0
Previous antineoplastic treatment (yes/no)								
	3/0	4/0	3/0	6/0	2/4	2/4	0/8	2/4

ECOG, Eastern Cooperative Oncology Group Performance Status Scale; vp, viral particles .

antibodies (NAbs titer), immune biomarkers (interferon (IFN)-γ, soluble lymphocyte activation gene-3 (sLAG3), interleukin (IL)-6, IL10), RNA sequencing (RNA-seq) analysis of tumor biopsies, and serum human hyaluronidase (PH20), overall response rate and PFS. A detailed description of the laboratory methods is shown in online supplemental appendix text 1.

Dose-escalation schedule

Only one dose of VCN-01 was administered to each patient during the trial, irrespective of whether they had been enrolled in Part I, Part II, or Part III. Online supplemental appendix figure 2 summarizes the dose-escalation schedule.

An accelerated dose scalation schedule was used in levels 1×10^{11} and 1×10^{12}. From level 3.3×10^{12} onwards, the study followed a modified Fibonacci dose-escalation schedule with commonly used definitions of DLTs. In parts II and III, the VCN-01 doses tested were the two highest tolerable doses from Part I. More details on the dose-escalation protocol are shown in online supplemental appendix figure 2. DLT was defined as any of the following criteria occurring during the period of the first cycle: (1) Hematological treatment-related adverse events

(AEs): any grade 4 neutropenia (absolute neutrophil count <0.5×10^9/L) lasting more than 5 days; neutropenic fever, any grade 4 neutropenia and sepsis or other severe infection, any grade 3 or 4 thrombocytopenia associated with bleeding. (2) Grade >2 cardiac or neurological toxicity. (3) Inability to tolerate the chemotherapy cycle due to toxicity. (4) Any toxicity, which in the opinion of the sponsor and investigator, was considered as DLT. (5) Any other grade 3–4 non-hematological treatment-related AEs, except for the following: grade 3 fatigue, nausea and vomiting, diarrhea, unless appropriate prophylactic or therapeutic measures had been administered; grade 3 elevation of hepatic transaminases lasting less than 7 days; non-clinically relevant biochemical abnormalities (ie, isolated increase of gamma-glutamyl transferase).

Statistics

Two population sets were defined for analysis: (i) safety population comprised all patients who had received the treatment dose of VCN-01; and (ii) an exploratory analysis of clinical activity was performed in those patients in the per-protocol population who had at least one evaluation of response performed at 8 weeks. Fisher's exact test was used to analyze the association between hyaluronic acid (HA) tumor staining and tumor response. One-way analysis of variance (Dunnett's multiple comparisons test) was used to determine the statistical significance of the different time points of PH20 concentration levels in serum. Paired Wilcoxon test was used to assess immune biomarkers variations and Spearman test was used to assess the association between tumor response and serum biomarkers levels. Non-linear regression analysis was used to fit a line and capture the relationships in a set of data and identify outliers. Kaplan-Meier estimations were used for survival analysis.

RESULTS

Patients

Baseline characteristics of the study population are listed in table 1. Thirty out of 114 patients screened were considered not eligible based on levels of NAbs, thus screening failure rate by this criterion was 26% (online supplemental appendix figure 3). In Part I, 16 patients (mostly colorectal, 15 metastatic colorectal cancer (mCRC) were treated with single-agent VCN-01 treatment at doses of 1×10^{11} viral particles (vp) (n=3), 1×10^{12}vp (n=4), 3.3×10^{12}vp (n=3) and 1×10^{13}vp (n=6). In Part II,12 patients with PDAC were treated with a single dose of VCN-01 (n=6 at dose 3.3×10^{12}vp and n=6 at dose 1×10^{13}vp) starting on day 1, followed by the standard-dose nab-paclitaxel plus gemcitabine chemotherapy regimen. In Part III, 14 patients with PDAC were treated with VCN-01 (n=8 at dose 3.3×10^{12}vp and n=6 at dose 1×10^{13}vp) administered on day 1 followed by nab-paclitaxel and gemcitabine regimen on day 8 (online supplemental appendix figure 4). Therefore, the safety population was comprised of 42 patients.

AEs, dose-escalation chronology, MTD and RP2D

Table 2 shows the list of adverse events according to grade and DLTs of parts I, II, and III. Detailed dose-escalation chronology is shown in online supplemental appendix text 2. The most frequent any grade event was fever/influenza like illness, overall and in each part of the study. The incidence of fever/influenza like illness was similar in all parts of the study (χ^2 p=0.47) and there was no association with viral levels in blood at 48 hours (Spearman p=0.49).

In Part I (n=16), 41.6% of TEAE were VCN-01-related, being fever (83%) the most frequent, followed by diarrhea (19%), vomiting (19%), musculoskeletal pain (19%) decreased appetite (19%) and aspartate aminotransferase (AST) increase (19%). VCN-01-related TEAEs were dose dependent and grade 4 TEAEs were observed only at 1×10^{13}vp in a single patient (AST and lipase increase). The RP2D was 1×10^{13}vp when VCN-01 was administered as monotherapy as only one out of six patients treated at 1×10^{13}vp experienced a DLT (subclinical grade 4 AST increase observed at day 5 post-VCN-01 that spontaneously resolved 2 days later).

In Part II (n=12), 26.9% of TEAE were VCN-01-related in combination with nab-paclitaxel and gemcitabine, and fever (67%) and thrombocytopenia (57%) were the most frequent. Grade 4 related TEAEs included thrombocytopenia (17%), febrile neutropenia (17%) and neutropenia (8%). Two DLTs were observed in two out of six patients treated at 1×10^{13}vp (one patient experienced a grade 4 febrile neutropenia—that appeared at day 7 after VCN-01 administration and resolved 7 days later—and another patient died due to an episode of enterocolitis and thrombocytopenia that initiated at day 1). Dose level 3.3×10^{12}vp was defined as the MTD/RP2D of VCN-01 in combination with nab-paclitaxel plus gemcitabine administered on day 1, in which none of the six patients treated suffered a DLT event.

In Part III (n=14), the most common TEAE related to VCN-01 combined with nab-paclitaxel and gemcitabine in the delayed schedule were fever (93%), vomiting (21%), asthenia (21%), nausea (21%) and alanine aminotransferase (ALT) increase (21%). No events qualified as DLTs. Therefore, the dose level 1×10^{13}vp was considered the RP2D of VCN-01 combined with nab-paclitaxel plus gemcitabine when administered with a 7-day shift (delayed schedule).

We measured IL-6 and IL-10 after VCN-01 intravenous injection as these cytokines had been previously associated to adenovirus toxicity.[11] We observed a~184 fold peak increase of IL-6 at 6 hours followed by a ~54 fold peak increase of IL-10 at 24 hours that do not associate with toxicity. Only a marginal association with toxicity grade is suggested at 48 hours (online supplemental appendix figure 5).

PKs and virus shedding

Data on PK for VCN-01 are shown in figure 1A. Dose linearity, as well as relevant VCN-01 exposure, were observed. Analysis of VCN-01 clearance in patients

Table 2 Adverse events

Preferred term	Grade (CTCAE)	Part I (n=16) No. of patients (%)	Part II (n=12) No. of patients (%)	Part III (n=14) No. of patients (%)
Fever/influenza like illness	Grade 1–2	12 (75)	8 (67)	12 (85)
	Grade 3–4	1 (6.3)	0	0
Asthenia	Grade 1–2	2 (12.5)	5 (42.0)	3 (21)
	Grade 3–4	0	1 (8.3)	0
Thrombocytopenia/platelet count decreased	Grade 1–2	1 (6.3)	3 (25)	2 (14)
	Grade 3–4	1 (6.3)	4 (33)*	0
Nausea	Grade 1–2	2 (12.5)	3 (25.0)	3 (21.4)
Vomiting	Grade 1–2	3 (18.8)	2 (16.7)	3 (21.4)
Musculoskeletal pain	Grade 1–2	3 (19)	4 (33)	0
Decreased appetite	Grade 1–2	3 (19)	3 (25.0)	0
Alanine aminotransferase increased	Grade 1–2	1 (6.3)	0	2 (14.3)
	Grade 3–4	1 (6.3)	0	1 (7.1)
Diarrhea	Grade 1–2	3 (18.8)	1 (8.3)	0
	Grade 3–4	0	0	1 (7.1)
Aspartate transaminase increased	Grade 1–2	1 (6.3)	0	0
	Grade 3–4	2 (12,5)	0	1 (7.1)
Neutropenia/neutrophil count decreased	Grade 1–2	2 (12.5)	1 (8.3)	0
	Grade 3–4	0	1 (8.3)	0
Headache	Grade 1–2	1 (6.3)	1 (8.3)	1 (7.1)
Hypotension	Grade 1–2	2 (12.5)	1 (8.3)	0
Febrile neutropenia	Grade 3–4	0	2 (16.7)	0
Hepatic enzymes increased	Grade 1–2	0	1 (8.3)	0
	Grade 3–4	0	1 (8.3)	0
Lipase increased	Grade 3–4	2 (12.5)	0	0
Amylase increased	Grade 1–2	1 (6.3)	0	0
	Grade 3–4	1 (6.3)	0	0
Dizziness	Grade 1–2	1 (6.3)	1 (8.3)	0
Arthralgia	Grade 1–2	2 (12.5)	0	0
Dyspnea	Grade 1–2	2 (12.5)	0	0
Enterocolitis	Grade 5	0	1 (8.3)*	0

VCN-01 related adverse events (PT) that occurred in more than one patient.
Coding was done with MedDRA Dictionary V.20.0. Worst case of severity is selected within a same patient.
*One patient enrolled in Part II developed concurrent grade 4 thrombocytopenia and grade 5 enterocolitis.
CTCAE, Common Terminology Criteria for Adverse Events.

enrolled in Part II did not show significant differences with respect to patients receiving VCN-01 as a single agent.

Shedding of VCN-01 was observed mostly until day 8 in all biologic fluids tested (urine, sputum, and feces), mainly in feces and sputum. VCN-01 genome levels in sputum and feces peaked by day 8, with 91% and 81% of positivity, respectively. Virus was no longer detected in most of the biological fluids from most patients by day 28 (online supplemental appendix figure 6). As sputum was considered the easiest route of horizontal transmission of VCN-01, sputum samples were analyzed for infectious viruses (anti-hexon staining), and no functional virus was found. There was no difference in the shedding profile of patients treated with VCN-01 alone or in combination with nab-paclitaxel/gemcitabine.

Viral replication after intravenous injection

Fourteen tumor biopsies were collected on days 8 or 28 from patients enrolled in parts II (nine patients) and III (five patients) to quantify the presence of the VCN-01 genome in tumor tissue by means of quantitative PCR (qPCR). The results are shown in figure 1B. On day 8, the presence of VCN-01 was demonstrated in five out of six subjects. In addition, E1A viral protein expression (a

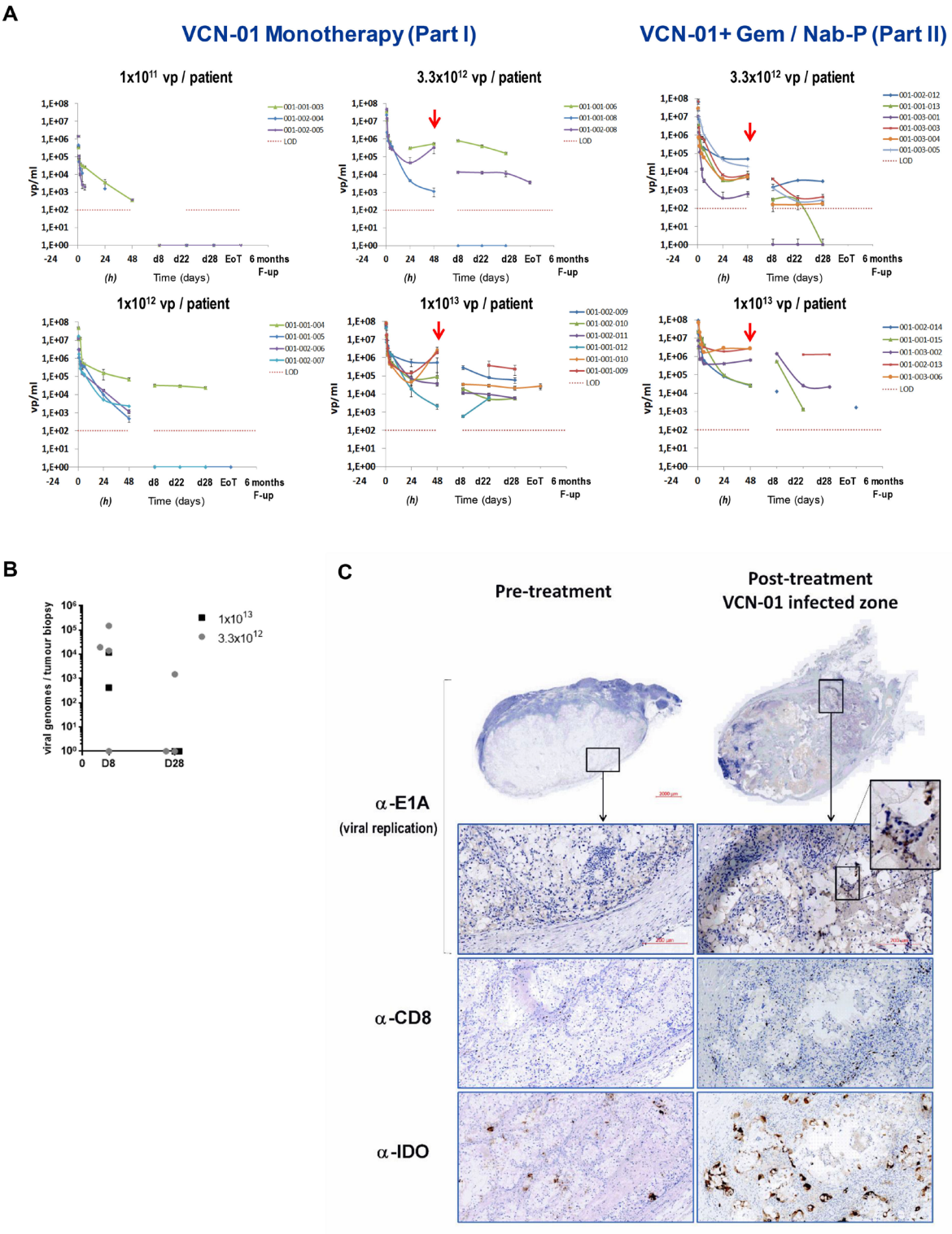

Figure 1 Pharmacokinetics and pharmacodynamics. (A) Pharmacokinetics. None of the patients treated at dose 1×10^{11} vp, and two out of four patients at dose 1×10^{12} vp showed VCN-01 genomes detected in blood from day 3 to 28. By contrast, most of the patients treated at the highest dose levels (3.3×10^{12} and 1×10^{13} vp) experienced secondary viremia peaks from 24 hours onwards and maintained VCN-01 genomes in blood for over 3 weeks after its administration. (B) Viral genome load in tumor biopsies. On day 8, the presence of VCN-01 was demonstrated in five out of six subjects (three out of four subjects in the 3×10^{12} vp level and both subjects in the 1×10^{13} vp level). One biopsy obtained from a lymph node metastasis in patient with pancreatic cancer 8 days after being dosed with VCN-01 at 3×10^{12} vp showed detectable expression of viral proteins in tumor cell nuclei. Positive staining was located adjacent to necrotic areas in the tumor biopsy. (C) Immunohistochemistry of E1A staining for viral replication colocalized with CD8 +in the tumor. VCN-01 also induced a potent inflammatory response, as evidenced by IDO upregulation within the same infected area (interferon- γ target). IDO, indoleamine 2,3-dioxygenase; vp, viral particles.

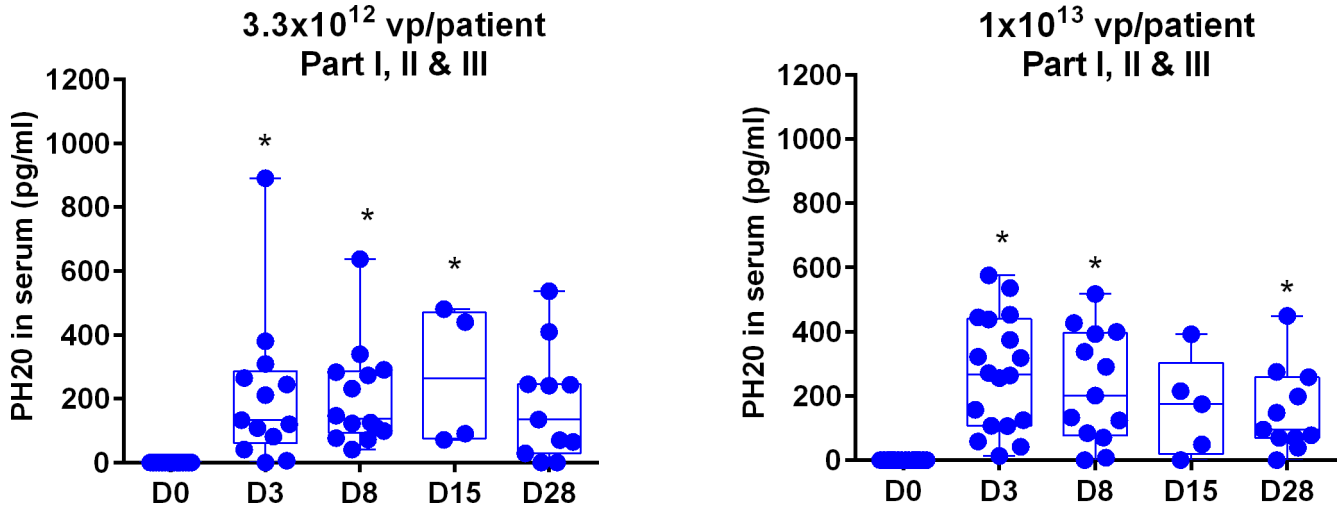

Figure 2 PH20 expression and detection in serum. PH20 expression from VCN-01 was measured on serum by ELISA in treated patients at different time points. PH20 expression in serum samples at different time points is expressed as pg/mL minus background levels detected for each sample at day 0 and represented in box and Whiskers graphs. An increase in hyaluronidase serum levels was detected in all 33 analyzed patients. One-way analysis of variance Dunnett's multiple comparisons test was statistically significant of the different time points (*p<0.05 vs D0). vp, viral particles.

non-capsid protein) was demonstrated by immunohistochemistry, confirming the active replication of VCN-01 in tumor biopsies (figure 1C). As PH20 is only expressed when VCN-01 can replicate, PH20 serum levels should be elevated if sufficient viral replication occurs. Accordingly, levels of PH20 in serum increased significantly in association with VCN-01 administration in all patients (n=33), peaking on day 3 and detected until day 28 (figure 2).

Systemic immune response

A panel of 34 immune markers containing cytokines, interleukins, soluble ligands, enzymes, and soluble immune checkpoint inhibitors were analyzed in serum samples of all patients to characterize the immunological-induced changes by VCN-01 as may have an impact on toxicity or activity (Parts I, II, and III) (online supplemental appendix figure 7A). VCN-01 increased pro-inflammatory mediators (as IL-6) immediately (6 hours on day 1), followed by IFN-γ, sLAG-3, interferon γ-induced protein 10 kDa (IP-10), indoleamine 2,3-dioxygenase (IDO1) and IL-10 that peaked at 24–48 hours. IFN-γ, IDO1, and IP-10 are markers of induced Th1 response. On day 8, peaks of IL-18 and soluble T cell immunoglobulin mucin domain 3 (sTIM-3) were observed. The immunological response normalized by day 28.

Baseline levels of anti-adenovirus Ad5 NAbs in patients before receiving VCN-01 ranged between 1/10 and 1/320 (median 1/80). After treatment, levels of anti-Ad5 NAbs increased from 64 to 32,800-fold, with a median of 1024.5-fold (online supplemental appendix figure 3).

Analysis of tumor biopsies

Exploratory tumor analyses were performed in a limited set of cases. Immunohistochemistry of E1A staining for viral replication colocalized with CD8 and IDO in the tumor (figure 1C), demonstrating a VCN-01 induced inflammatory response within the tumor microenvironment. This VCN-01 mediated inflammatory response was further confirmed in available biopsies. CD8 infiltration was increased in 54% of the biopsies (6/11) and Tregs decreased in 40% of biopsies (FoxP3 and CD25 staining) (4/10). The programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) axis was also upregulated (67% for PD-1 (8/12) and 17% for PD-L1 (2/12)). CTLA-4 staining was also increased (40%, 4/10) and most biopsies showed increased IDO upregulation (67%, 8/12). Gene set Enrichment Analysis of RNA-seq data of paired pre-treatment and day 8 post-treatment samples from five patients with PDAC pointed to increased enrichment of several pathways, most notably those related to extracellular matrix organization, collagen formation, and integrin signaling. In addition, there was an upregulation of CD28, PD-1, and CTL signatures (online supplemental appendix figure 8 and table 1). Altogether these results confirm that virus targeting promotes a clear change in the tumor immune environment.

Preliminary analysis of clinical activity

This phase 1 study was not designed to assess efficacy, but clinical activity was preliminary evaluated for each arm.

Part I: Four out of 16 subjects (one in the 1×10^{11} vp dose group, two in the 1×10^{12} vp dose group, and one in the 1×10^{13} vp dose group) had stable disease as the best response. No partial or complete responses were observed.

Part II: Ten out of 12 patients were evaluable for tumor response, and the overall response rate 50% (5 out of 10). No patient experienced tumor progression at the 8-week evaluation. All patients achieving an objective response benefitted from long stabilizations, lasting more than

1 year, and one of them experienced prolonged survival of more than 4 years. No differences in response rate were observed between the 3.3×10^{12} vp and 1.0×10^{13} vp dose groups. Median PFS was 9.9 months (2.8–19.9) and median OS (post-hoc analysis) was 11.0 months (5.0–48.4). In addition, delayed responses were observed in two patients at weeks 32 and 56. Five patients (50%) survived more than 12 months.

Part III: Twelve out of 14 patients were evaluable for tumor response. Partial response was observed in 6 out of 12 patients (50%), one out of six at 3.3×10^{12} vp, and five out of six at 1.0×10^{13} vp. Three patients (25%) experienced disease stabilization lasting more than 1 year. Median PFS was 6.7 months (1.6–14.4), and median OS (post-hoc analysis) was 13.5 months (2.6–29.6+). Figure 3 shows the changes in tumor burden in parts II and III in the efficacy population. One additional patient in Part III benefited from a delayed response evident at week 48 after treatment initiation. Eight patients (66.7%) survived more than 12 months. In addition, a subgroup analysis of patients at the RP2D (1.0×10^{13} vp/patient followed by nab-paclitaxel plus gemcitabine 1 week later, n=6) showed an overall response rate (ORR) of 83%, with a median PFS of 6.3 months and median OS of 20.8 months.

Post-hoc analysis of the activity of patients included in Part II and III showed a 50% ORR in the 22 evaluable patients treated with VCN-01 in combination with nab-paclitaxel plus gemcitabine, respectively, with eight prolonged stabilizations lasting more than 1 year. Median PFS was 7.2 months, and median OS was 13.4 months. Figure 3C shows the waterfall plot of Part II and III pooled analyses. Data on antitumor activity are summarized in table 3 and figure 3.

To identify potential biological markers of VCN-01 associated with clinical activity, associations were explored. Baseline levels of NAbs did not associate with tumor response (online supplemental figure 3B,C). However, there was an association between the highest NAbs fold change after VCN-01 treatment with tumor reduction and response (online supplemental figure 3D,E). Higher levels of IFN-γ (24 hours) and sLAG3 (48 hours) may be associated with greater tumor reduction (online supplemental appendix figure 7B,C). Higher peak sera levels of PH20 were associated with maximum tumor shrinkage, suggesting that responding patients had higher replication of VCN-01 measured as PH20 expression (online supplemental appendix figure 9).

DISCUSSION

The results of this study show that the treatment of patients with cancer with intravenous VCN-01 oncolytic adenoviruses is feasible and is associated with expected and manageable AEs. Intravenous VCN-01 has demonstrated a good tolerability profile, in line with other Ad5-based oncolytic viruses (24), and its MTD in monotherapy was the maximal feasible dose (1×10^{13} vp/patient) which is the highest RP2D reported among different clinical trials in the field of oncolytic adenoviruses. This dose is considered the RP2D of VCN-01 combined with nab-paclitaxel plus gemcitabine when administered with a 7-day shift (delayed schedule).

The clinical experience with oncolytic adenoviruses is significant as more than 42 clinical trials have been performed in different cancer indications. However, less than 25% of the trials evaluated intravenous administration of the virus.[12] A dose-escalating study with oncolytic adenovirus Onyx-015 found no dose limiting toxicity, doses ranged from 2×10^{10} to 2×10^{13} vp/patient. However the highest dose of 2×10^{13} vp/patient was only administered to one patient and therefore the dose selected in subsequent phase II studies was at 2×10^{12}vp/patient; patients administered ≥2×10^{12} vp/patient showed fever, rigors and transient transaminase elevations.[13] In a subsequent phase II study, Onyx-015 was intravenously administered at 2×10^{12} vp/patient on days 1 and 15 of 28-day cycles for six cycles. Most patients (83%) had influenza-like symptoms (fever, fatigue, chills).[14] A higher viral dose at 6×10^{12} vp/patient was evaluated with CV-706 (CG7870) by systemic administration in hormone-refractory metastatic prostate cancer also made patients experienced influenza-like symptoms, grade 2 transaminitis and/or isolated D-dimer elevations.[11] Another oncolytic adenovirus ICOVIR-5 was also administered up to 1×10^{13} vp/patient, but two DTLs (transaminitis) were found and the RP2D was set at 3.3×10^{12} vp/patient.[15] With enadenotucirev, a group B Ad11p/Ad3 chimeric oncolytic adenovirus, the systemic MTD was established at 3×10^{12} vp/patient, doses at 6×10^{12} and 1×10^{13} vp/patient were discarded due to toxicities (hypoxia and transaminitis), and the most frequent AEs were fever and chills, but also grade ≥3 hypoxia, lymphopenia, and neutropenia.[16] Although the combination with nab-paclitaxel plus gemcitabine in Part II increased the rate of neutropenia and thrombocytopenia, the delay of 7 days between VCN-01 and nab-paclitaxel plus gemcitabine administration allowed spanning chemotherapy toxicities from viral ones, and the MTD and RP2D for Part III was the same that for Part I at 1×10^{13} vp/patient.

The main drawback of the systemically administered oncolytic adenoviruses tested so far has been the limited tumor targeting. The high prevalence of pre-existing anti-Ad5 NAbs in the general population has been considered a major hurdle for systemic efficacy of gene and oncolytic therapies employing human adenovirus type 5 (Ad5).[17] Accordingly, only patients with low anti-Ad5 NAbs titers (<1/350 dilution) were considered eligible and 26% of screened patients were excluded due to this criterion. In addition, very high doses of VCN-01 were administered so that even if some viruses are neutralized, VCN-01 could still reach the tumor. VCN-01 tumor targeting was confirmed in our trial and was similar to that observed by the non-Ad5 oncolytic adenovirus enadenotucirev (Ad11 capsid based) that has low seroprevalence in humans.[18 19] In these selected patients (anti-Ad5 titers <1/350 dilution) no correlation was observed between

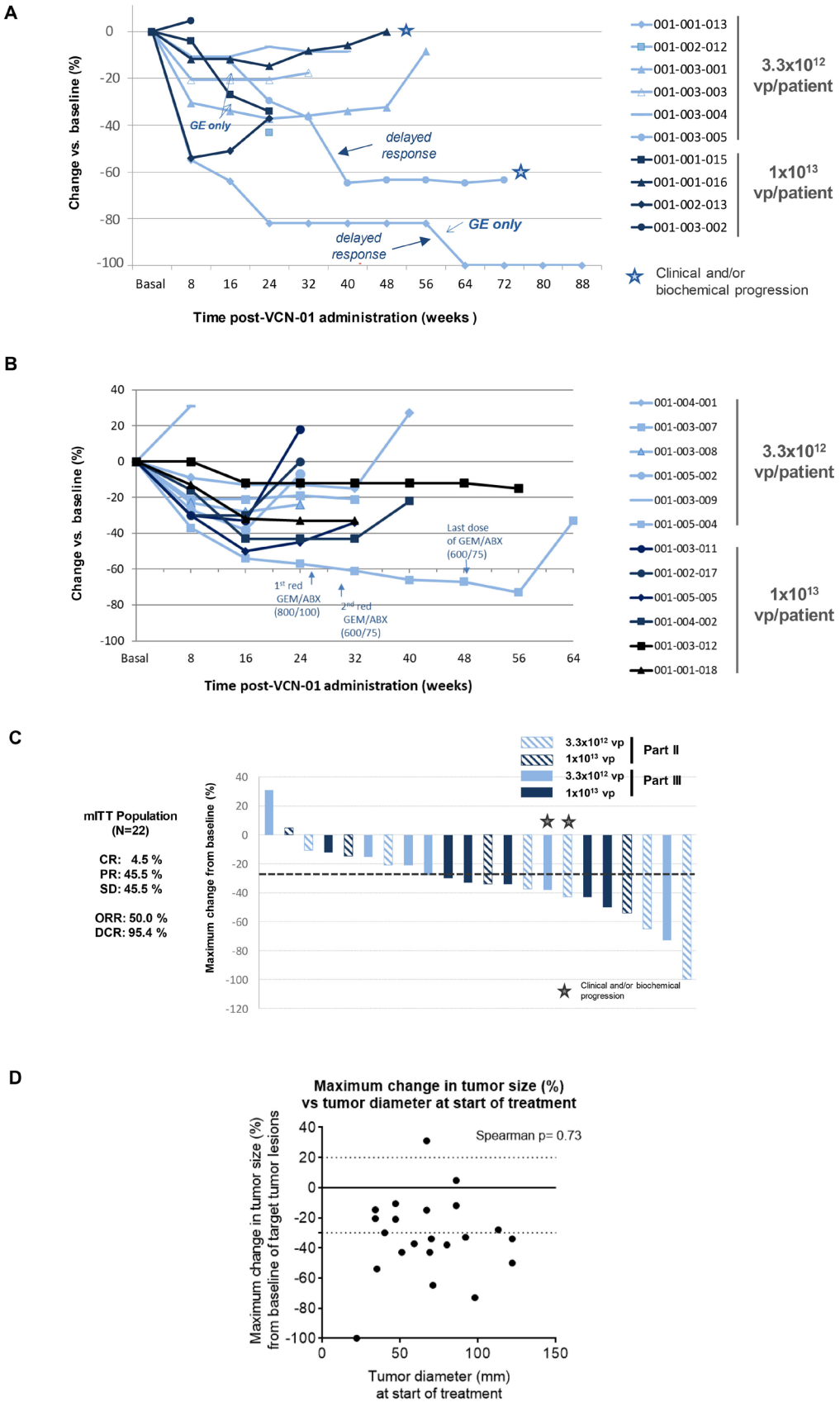

Figure 3 Antitumor effects of VCN-01. (A) Part II. Change in tumor burden in patients receiving concurrent VCN-01 and nab-paclitaxel plus gemcitabine. (B) Part III. Change in tumor burden in patients receiving delayed VCN-01 and nab-paclitaxel plus gemcitabine. (C) Waterfall plot of tumor burden changes in patients included in Parts II and III of the study. (D) Maximum change in tumor size (%) versus tumor diameter at start of treatment showed no association between starting tumor size with antitumor efficacy. CR, complete response; ORR, overall response rate; PR, partial response; SD, stable disease; vp, viral particles.

Table 3 Clinical activity

	Part II		Pooled Part II and III
	N=10 (Efficacy population)	Part III N=12	N=22 (Efficacy population)
Response rate	**N (%)**	**N (%)**	**N (%)**
Complete response	1 (10)	0	1 (4.5)
Partial response	4 (40)	6 (50)	10 (46)
Stable disease (SD)	5 (50)	6 (42)	10 (46)
Overall response rate (ORR)	5 (50)	6 (50)	11 (50)
Long duration SD*	5 (50)	3 (25)	8 (36.4)*
ORR at 3.3×10^{12}	3 (50)	1 (17)	4 (33.3)
ORR at 1.0×10^{13}	2 (50)	5 (83)	7 (70)
Survival analyses	**Median in months (95% CI)**	**Median in months (95% CI)**	**Median in months (95% CI)**
Progression-free survival (PFS)	9.94 (2.83 to 19.85)	6.69 (1.6 to 14.4)	7.22 (1.6 to 19.85)
Overall survival	11.0 (5.03 to 48.43)	13.47 (2.6 to 29.6+)	13.35 (2.6 to 48.43)

*(>1 year).

baseline NAbs levels and antitumor response. As anti-Ad5 levels were very much increased after a single injection, a second intravenous administration was discarded. In addition, compared with previous clinical trials, Onyx-015 only showed tumor targeting in one patient,[13] ICOVIR-5 showed more frequent tumor targeting (36%),[15] and consistent tumor targeting has been observed with enadenotucirev (most samples)[18] similar to that observed with VCN-01 in this study. The comprehensive VCN-01 PK and pharmacodynamic evaluation performed, points to viral targeting and virus replication in tumor tissue after a single intravenous injection. The second viremia peaks and the variations of PK parameters in a dose-dependent way are consistent with virus replication. Detection of the virus in tumor biopsies (as assessed by E1A early viral protein and qPCR), and kinetics of hyaluronidase in sera further confirm it. Taken together, all these data strongly suggest that VCN-01 can be safely administered intravenously at higher doses ($\geq3.3\times10^{12}$ vp/patient) that overcomes the Kupffer threshold in humans resulting in viral exposure and replication within the tumor.

A second intravenous administration of VCN-01 was not considered in this study as high levels of anti-Ad5 neutralizing antibodies were observed after the first administration. We are currently monitoring the anti-Ad5 NAbs titers over longer periods of time to identify when such levels are low enough to allow a second intravenous administration. In a separate clinical trial, VCN-01 was repeatedly administered directly into PDAC tumors (IT) because the impact of NAbs with this type of delivery is minimal[20] although, through this type of administration viral biodistribution is restricted to the injection site and metastatic sites are not infected. Repeated intravenous administration, if feasible, will offer better biodistribution of the virus within each tumor mass and has the potential to reach all metastatic tumor sites.

The added effect of VCN01 to the chemotherapy regimen cannot be elucidated in a non-randomized setting. Yet, early clinical analysis shows encouraging activity signals with a response rate of 50% and a median OS of 13.4 months when VCN-01 was combined with nab-paclitaxel plus gemcitabine that deserve to be further explored.

In pancreatic cancer the only other reported systemic treatment with an oncolytic virus is Reolysin (an oncolytic reovirus) in combination with gemcitabine which had one partial response (3%) and 16 stable disease (55%).[21] VCN-01 has shown oncolytic activity and synergistic effects when combined with nab-paclitaxel plus gemcitabine in preclinical models of pancreatic ductal adenocarcinoma.[20] Also, a phase I clinical trial of intratumoral administration of VCN-01 in patients with pancreatic ductal adenocarcinoma demonstrated local tumor control and the modification of tumor matrix stiffness when administering VCN-01.[20]

A transient systemic immune response was induced by VCN-01. Our exploratory data suggested that IFN-γ and sLAG-3 increases due to VCN-01 correlated with a reduction in tumor size. IFN-γ has been previously associated as a biomarker of immunotherapies efficacy,[22] whereas the serum sLAG3 has been recently used as predictive biomarker in breast and gastric cancers.[23 24]

One of the key essential modifications of VCN-01 is the expression of a soluble form of human recombinant hyaluronidase (PH20). Recent data from two separate clinical trials evaluating systemic PEGPH20 combined with nab-paclitaxel plus gemcitabine or FOLFIRINOX in patients with advanced pancreatic ductal adenocarcinoma were negative.[25 26] The aim of these PEGPH20 trials was to increase intratumoral delivery of chemotherapy by destroying the HA by the systemic administration of PEGPH20, a pegylated form of recombinant human hyaluronidase.However, VCN-01 has a different

therapeutic strategy, combining the local tumor expression of PH20 with intrinsic oncolytic effects, and the induction of inflammation by both the viral infection as well as cancer cell death. VCN-01 demonstrated direct cancer cell killing (E1A staining), thereby attracting the immune system (CD8 +lymphocytes infiltration) and inflaming the tumor cells (measured by IDO expression). Our preliminary clinical observations suggest that local tumor expression of PH20 does not associate with the limitations observed with systemically administered PEGPH20. Previous reports have shown dense stroma as a barrier for drug delivery in pancreatic ductal adenocarcinoma,[27–29] and its degradation by hyaluronidase as an effective method to overcome this issue.[30 31] Preclinically, VCN-01 has also demonstrated enhanced delivery of chemotherapy and therapeutic antibodies into the tumor.[20] A putative advantage provided by the virally-expressed hyaluronidase would be the combined local expression of PH20 for approximately 28 days with an intrinsic oncolysis resulting in tumor debulking and induction of intratumor inflammation. VCN-01 has also recently demonstrated to reduce tumor stiffness in patients with PDAC treated via intratumoral administration which suggests that VCN-01 replicates within the tumor mass and induces stromal disruption.[20] Of note, higher levels of hyaluronidase detected in patients' serum may be associated with a more pronounced tumor response in patients. In this line, stromal disruption may be only the initial mechanism that enables the induction of additional long-lasting mechanisms. The synergistic effect between VCN-01 and nab-paclitaxel plus gemcitabine in pancreatic ductal adenocarcinoma cell lines has also been observed in two different animal models (mice and hamster).[20] Moreover, patients' tumor RNA-seq analysis showed enrichment of extracellular matrix organization pathways after VCN-01-treatment. Altogether, these data suggest VCN-01 mediated antitumor activity through viral replication and PH20 expression.

Of note, we observed that some VCN-01 treated patients benefitted from late-onset responses. This form of delayed antitumor activity is not common with chemotherapy. However, it is more frequent in patients treated with immunotherapy in whom the onset of response can be observed even after discontinuation of treatment.[32] An immune mechanism of action associated with the oncolytic activity of VCN-01 may be the underlying explanation. VCN-01 induced a potent inflammatory environment (IDO, CD28, PD-1, CTL signature upregulation, and collagen formation) after treatment, in the predominantly immune-desert pancreatic adenocarcinomas. VCN-01 E1A protein expression and CD8 +lymphocytes were also found in the same tumor area, in agreement with viral replication. E1A was detected adjacent to necrotic areas in the tumor, suggesting an association between VCN-01 replication and tumor necrosis. We hypothesize that the antiviral immune mediated responses could also induce antitumor mediated responses when the virus replicates within the tumor.

VCN-01 may be added to the short list of agents that have been combined with the doublet nab-paclitaxel plus gemcitabine to achieve improved outcomes in advanced PDAC. To date, the results of this strategy have had different outcomes. While hydroxychloroquine failed to show clinical benefit,[33] some combinations seem to be promising, such as those with the hedgehog inhibitor vismodegib[34] or with cisplatin.[35] Furthermore, the preclinical data from a model of pancreatic ductal adenocarcinoma indicate that hyaluronidase activity associated to intravenous delivery of VCN-01 increases both extravasation and tumor uptake of an anti-PD-L1 antibody,[20] which opens another avenue for developing combinations of VCN-01 and checkpoint inhibitors in the future.

In conclusion, VCN-01 is a next generation oncolytic virus successfully designed for retargeting tumors. We confirm its oncolytic viral replication competence after intravenous administration to patients with cancer. When combined intravenously with nab-paclitaxel plus gemcitabine in patients with PDAC it results in a safe profile and encouraging clinical and biological activity that deserves further investigation.

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