Phase I/IIa Trial in Advanced Pancreatic Ductal Adenocarcinoma Treated with Cytotoxic Drug-Packaged, EGFR-Targeted Nanocells and Glycolipid-Packaged Nanocells

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ABSTRACT

Purpose: We assessed the safety and efficacy of an EGFR-targeted, super-cytotoxic drug, PNU-159682-packaged nanocells with α-galactosyl ceramide-packaged nanocells (E-EDV-D682/GC) in patients with advanced pancreatic ductal adenocarcinoma (PDAC) who had exhausted all treatment options.

Patients and Methods: ENG9 was a first-in-man, single-arm, open-label, phase I/IIa, dose-escalation clinical trial. Eligible patients had advanced PDAC, Eastern Cooperative Oncology Group status 0 to 1, and failed all treatments. Primary endpoints were safety and overall survival (OS).

Results: Of 25 enrolled patients, seven were withdrawn due to rapidly progressive disease and one patient withdrew consent. All 25 patients were assessed for toxicity, 24 patients were assessed for OS, which was also assessed for 17 patients completing one treatment cycle [evaluable subset (ES)]. Nineteen patients (76.0%) experienced at least one treatment-related adverse event (graded 1 to 2) resolving within hours. There were no safety concerns, dose reductions, patient withdrawal, or treatment-related deaths.

Median OS (mOS) was 4.4 months; however, mOS of the 17 ES patients was 6.9 months [208 days; range, 83–591 days; 95.0% confidence interval (CI), 5.6–10.3 months] and mOS of seven patients who did not complete one cycle was 1.8 months (54 days; range, 21–72; 95.0% CI, 1.2–2.2 months). Of the ES, 47.1% achieved stable disease and one partial response. Ten subjects in the ES survived over 6 months, the longest 19.7 months. During treatments, 82.0% of the ES maintained stable weight.

Conclusions: E-EDV-D682/GC provided significant OS, minimal side effects, and weight stabilization in patients with advanced PDAC. Advanced PDAC can be safely treated with super-cytotoxic drugs via EnGeneIC Dream Vectors to overcome multidrug resistance.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death worldwide with a poor prognosis and a 5-year survival rate of 10% or less (1). Due to nonspecific or vague symptoms when the cancer is still localized, most patients are diagnosed with advanced-stage metastatic disease (2).

Surgical resection is presently the only therapeutic modality, however, only about 20% of patients with PDAC have resectable cancers and the recurrence rate after surgery is as high as 85% (3). Multiagent chemotherapy with drugs like gemcitabine, FOLFIRINOX, and nab-paclitaxel are treatments of choice, but resistance to therapy prevails (4, 5). Median overall survival (OS) is still modest between 6.7 months (gemcitabine) to 11.1 months (FOLFIRINOX; refs. 6, 7). Unfortunately, due to grade 3 and 4 toxicities, FOLFIRINOX is restricted to patients with a good Eastern Cooperative Oncology Group (ECOG) performance status (6, 8).

We have developed cytoimmunotherapy for the targeted treatment of a range of solid tumors. In brief, it is a bacterially derived, 400-nm diameter, achromosomal, nonliving nanocell [designated EnGeneIC Dream Vector (EDV)] that can be packaged with a range of different chemotherapeutic drugs or adjuvants, specifically targeted to tumor cells via single-chain bispecific antibodies attached to an EDV-surface O-polysaccharide component of lipopolysaccharide (LPS; **Fig. 1A**). Due to its large size, it does not extravasate into normal tissues postintravenous administration, and EDV exit specifically into the tumor microenvironment via tumor-associated leaky vasculature. EDV engage the tumor cell surface receptor via the targeting antibody and are macropinocytosed by tumor cells, broken down in lysosomes, resulting in intracellular release of the drug (9–11). EDV are also phagocytosed by macrophages and dendritic cells activating an innate and adaptive tumor-specific immune response, which enhances antitumor activity (9–11).

PNU-159682 is a secondary metabolite of nemorubicin (MMDX) and is more potent than either MMDX (800- to 2400-fold) or doxorubicin (2100- to 6400-fold; ref. 12). Due to its extreme potency, it cannot be used in conventional chemotherapy and is currently being explored for development of antibody–drug conjugates (13). In previous studies, we had analyzed biopsy-derived

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Translational Relevance

Chemotherapies available for treatment of advanced metastatic pancreatic ductal adenocarcinoma (PDAC) are limited to a few drugs, which have been used for decades, like gemcitabine, paclitaxel, irinotecan, folinic acid, and oxaliplatin. Unfortunately, PDAC tumor cells rapidly become multidrug resistant, and patients run out of treatment options resulting in an abysmal overall survival (OS). Here, we show in a first-in-man phase I/IIa clinical trial in 17 patients with advanced metastatic PDAC who had exhausted all treatment options that a super-cytotoxic drug, PNU-159682, packaged in EnGeneIC Dream Vector nanocells and targeted via bispecific antibodies directed to EGFR could be administered in these patients (50 to 75 repeat doses) to overcome multidrug resistance, achieve disease stabilization/regression, stabilize body weight, and significantly increase OS with minimal to no toxicity. This opens a way to safely and effectively deliver a range of new super-cytotoxic drugs to overcome multidrug resistance in end-stage cancers.

PDAC tumor cells in MTS (cell proliferation) assays, and had shown that these cells exhibited multidrug resistance and were highly resistant to all conventional anticancer drugs like gemcitabine, 5-fluorouracil, irinotecan, abraxane, oxaliplatin, etc. However, these cells were highly sensitive (IC_{50} in low nM range) to super-cytotoxic drugs like PNU-159682 (11).

Therefore, for this study, where there was intention to treat patients with advanced PDAC with overexpression of EGFR on tumor cell surface (14–16), PNU-159682-packaged, EGFR targeted EDV (designated as E-EDV-D682) were selected to address multidrug resistance.

In this study, we also demonstrated that EDV packaged with glycolipid iNKT cell-stimulating adjuvant α-galactosyl ceramide when added to E-EDV-D682 could augment antitumor activity.

Therefore, both EDV in appropriate concentrations were used in this study (the combination designated as E-EDV-D682/GC; **Fig. 1A** and **B**).

This study is a phase I/IIa, first-in-man clinical trial carried out in patients with advanced PDAC who had exhausted all treatment options or where other available treatments were not appropriate due to patient comorbidities. The results here demonstrate that a super-cytotoxic drug (PNU-159682) packaged in EDV nanocells can be administered in patients with PDAC with minimal toxicity. While the patient numbers are small, early signs of antitumor efficacy in terms of tumor stabilization/regression and OS with weight loss stabilization were achieved.

Patients and Methods

Study design and patient eligibility

Carolyn trial (ENG9; Australian New Zealand Clinical Trials Registry; ANZCTR, ACTRN12619000385145) was a single-arm, open-label, phase I/IIa, dose-escalation clinical trial conducted at two medical cancer centers, Frankston Private Hospital, Melbourne and Adventist Hospital, Sydney, Australia.

Eligible patients (aged ≥ 18 years) had histologically confirmed diagnosis of PDAC, disease progression (**Table 1**) with measurable lesions according to Immune Response Evaluation Criteria in Solid Tumors (iRECIST; v1.1; ref. 17), an ECOG performance status of 0 to 1, life expectancy of at least 12 weeks, adequate hematologic, hepatic, and renal function, and had failed all treatment options or available treatment options deemed not appropriate in these patients. EGFR expression on archived or tumor biopsies was confirmed by IHC.

Exclusion criteria for patients included (i) investigational therapy, radiotherapy, or major surgery, 28 days prior to the EDV dose, (ii) significant pericardial effusions, pleural effusions, or ascites, (iii) concurrent unstable diabetes mellitus, (iv) coronary artery disease, congestive heart failure, uncontrolled hypertension or cardiac arrhythmia, (v) clinically significant electrocardiogram changes, which obscure the ability to assess the PR, QT, and QRS interval, congenital long QT syndrome, (vi) human immunodeficiency virus, hepatitis B or C positive, (vii) uncontrolled arterial or venous thrombosis, (viii) active or uncontrolled severe infection, (ix) other clinically significant disorders that, in the opinion of the investigator would pose a risk to subject safety or interfere with the study evaluation, procedures, or completion.

All patients provided written informed consent, and the study was conducted in accordance with the International Conference on Harmonisation Good Clinical Practice guidelines, annotated by the Australian Therapeutic Goods Administration (2018), the Australian National Health and Medical Research Council (NHMRC) Statement on Ethical Conduct in Human Research (2018), and the Declaration of Helsinki (1964) with seventh revision (2013). This study was approved by Bellberry Human Research Ethics Committee (HREC), Australia (Approval No. 2018–11–1008).

Treatment procedures

The study explored five dosing regimens of E-EDV-D682/GC (**Table 2** and details in Supplementary information Table S1), each with an intracycle dose-escalation schedule.

The maximum tolerated dose (MTD) of EDV regardless of payload of chemotherapeutic drug or nucleic acid was determined previously from multiple phase I clinical trials, and this was found to be 8×10^9 EDV per dose (18–21). All these trials had explored treatment cycles that comprised a single EDV dose per week for 7 weeks followed by radiologic examination during week 8. In addition, all EDV doses were administered intravenously as a 20-mL infusion (EDV suspended in water for injection) over 20 minutes using an automated pump.

Hence, this trial explored five different dosing regimens to determine the safety of: (i) EDV dosing twice a week, (ii) shorter cycle times of 4 or 5 weeks, (iii) EDV dosing twice a visit (45 minutes apart) twice per week, (iv) EDV dosing three times a visit (45 minutes apart) twice per week, and (v) bolus EDV doses instead of a 20-minute infusion.

Regimens 1 and 2 were over 7 weeks with radiologic evaluation during week 8. For regimens 1 and 2, the maximum dose of E-EDV-D682/GC was 5.5×10^9 and 8×10^9, respectively.

Regimen 3 explored a more aggressive approach of dosing twice each visit (45 minutes apart), biweekly for the first 2 weeks followed by weekly for an additional 2 weeks, and shortening the cycle time to 5 weeks.

For regimens 1 to 3, the EDV doses were administered as in previous phase I studies as a 20-mL infusion over 20 minutes.

Regimens 4 and 5 explored the safety of bolus EDV doses with regimen 4 administering two doses (45 minutes apart) twice weekly for 4 weeks and regimen 5 being the same as regimen 4 except for administering three EDV doses (45 minutes apart) during each visit.

The first three patients recruited to each regimen were reviewed for safety by the Safety Review Committee (SRC). When the test product was well tolerated and there were no safety concerns, the SRC would

recommend the study to move forward with an additional three patients in each regimen followed by commencement of the next regimen. Dose reduction was available if dose limiting toxicities (DLT) were observed at any dose level.

Participants continued the treatment unless the subject became intolerant to the study treatment, withdrew consent, or if in the opinion of the investigator, the individual was no longer benefiting (factors in consideration were disease progression radiologically or clinically).

Outcomes

For all the regimens, the following safety and efficacy variables were monitored during the study: (i) medical history, (ii) concomitant medication, (iii) weight and height, (iv) vital signs, (v) ECOG performance status, (vi) 12-lead ECG, (vii) echocardiogram, (viii) full blood hematology and biochemistry, (ix) C-reactive protein (CRP), (x) serum inflammatory cytokines, anti-LPS antibodies, (xi) urinalysis, and (xii) MRI or CT (± PET) scans to assess disease burden. At the end of each cycle, tumor response was assessed by radiologic examination, including CT or MRI based on iRECIST criteria.

Assessments

The primary endpoints of this study were (i) toxicity, (ii) initial efficacy evaluation, including tumor response and (iii) OS. Secondary objectives included progression-free survival (PFS) and cytokine response.

Safety assessment

Safety outcomes were presented by adverse events (AE), including serious AE (SAE), DLT, and clinically significant laboratory findings, including inflammatory responses. AE were graded according to the CTCAE (version 5.0), and any AE with severity ≥ grade 3 were classified as severe AE. Treatment toxicity was evaluated continuously throughout the study and during follow-up.

The first three evaluable participants recruited to each regimen were reviewed for safety by the SRC. When the test product was well tolerated and there were no SAE having any causal relationship to the test product, and no DLT was identified, the SRC would recommend the study to move to the next regimen.

OS and PFS

Upon completion of the study, patients were followed up for survival until death. OS was calculated from the date of first administration of treatment to the date of death regardless of cause. PFS was calculated from the date of first administration of treatment to the date of clinical disease progression or death regardless of cause, whichever occurs earlier.

Mouse allograft studies

All animal work was approved and performed according to the guidelines set out by the EnGeneIC's Animal Care and Ethics Committee under AEC 03/2021. These guidelines comply with the NHMRC Australian Code for the care and use of animals for scientific purposes.

Female C57BL/6 mice were subcutaneously injected in the left flank with KPC 1242 murine pancreatic cancer cells (passage 6–10), kindly provided by Professor David Tuveson, Cold Spring Harbor Laboratory Cancer Center, New York. The cell line was isolated from PDAC tumor tissues obtained from LSL-$Kras^{G12D}$; LSL-$Trp53^{R172H}$; Pdx1-Cre mice of a pure C57BL/6 background (22). Cells were tested for *Mycoplasma* by PCR/RT-PCR (Cerberus Sciences; Victoria) and the last negative test obtained in December 2021 prior to cell implantation.

Treatments were administered via tail vein injection when tumors were ∼150 mm^3. Mice were treated with the following treatments: saline, 1×10^9 EDV only, 5×10^6 EDV-αGC, 1×10^9 mEGFR-EDV-682, and combined EDV-αGC (5×10^6) + mEGFR-EDV-682 (1×10^9). Mice were injected three times a week for 2 weeks. Tumors were measured three times a week using a caliper and tumor volume in mm^3 was calculated as length × $width^2$ × 0.5.

Enriched CD8α+ T cells were obtained from single-cell suspensions of spleens using the mouse CD8α+ T Cell Isolation Kit (Miltenyi Biotec). Mouse iNKT cell isolation from spleen was conducted using NKT14 antibody according to the method previously described (23). Isolated $CD8^+$ T and iNKT cell cytotoxicity were evaluated against KPC 1242 tumor cells and cell growth/survival was monitored using xCELLigence real-time cell analysis system (RTCA, ACEA Biosciences).

Statistical comparison in the tumor regression study was performed using one-way ANOVA. The level of significance is expressed as follows: ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. All statistical analyses were performed using GraphPad Prism, RRID:SCR_002798.

In other *in vitro* studies, we had demonstrated that postlysosomal breakdown of αGC-packaged EDV (EDV-GC), the released αGC is displayed on the surface of dendritic cells via the MHC class I-like molecule CD1d, and this complex is recognized by the invariant NKT cell surface receptor, which then triggers the activation of iNKT cells (24). Mouse tumor dissection studies had shown that the enhanced antitumor efficacy was likely due to the rapid infiltration of tumor-specific CD8+ T cells as well as iNKT cells.

Cytokine assays

Serum cytokines, including interleukins IL6, IL8, IL10, and TNFα were measured using DuoSet ELISA kit from R&D Systems according to the manufacturer's instructions. IFNγ was measured using Quantiline HS ELISA Kit (R&D Systems) based on the manufacturer's instructions. Cytokine concentration was determined by absorbance against standard curve and expressed in pg/mL.

Statistics

Data were summarized by the following descriptive statistics: *n* (number of observations), mean, SD, median, minimum, maximum. Categorical data were summarized by frequencies and percentages. The 95.0% confidence intervals (CI) were provided.

Data availability

All clinical and preclinical data included in this study are available upon reasonable request from the corresponding author. The data generated in this study do not comprise genomics, sequencing, genotype/phenotype, clinical variation, crystallography, macromolecular structure, nor new code required for repository.

Results

Payload concentration in EDV

Spectrophotometric quantitation of PNU-159682 extracted from E-EDV-D682 showed that approximately 378 ng of PNU was packaged in 10^9 E-EDV-D682 (Supplementary Table S2). Purified EDV were loaded with αGC to produce αGC-EDV, and LC/MS-MS measurement showed approximately 70 ng of αGC per 10^9 EDV (Supplementary Table S3).

Mouse allograft studies

Mouse allograft study with KPC 1242 cells showed that mice treated with mEGFR-EDV-D682 plus EDV-αGC provided maximal antitumor efficacy compared with controls without EDV-αGC (**Fig. 1C**). When the CD8+ T cells and iNKT cells from the spleen of the variously treated mice were analyzed in the xCELLigence assay (25), the results showed that the CD8+ T cells from mEGFR-EDV-D682 plus EDV-αGC were able to kill KPC 1242 cells (**Fig. 1D**) but not as effectively as the iNKT cells (**Fig. 1E**), which showed sustained killing of KPC 1242 cells over 30 hours. These results suggested that the stimulation of both tumor-specific CD8+ T cells and iNKT cells are more effective in antitumor efficacy than either cell type alone.

Patient demographics and prior therapies

Between February 18, 2019, and March 2, 2022, 25 patients with advanced PDAC who had exhausted all treatment options or where approved available treatments were not appropriate due to comorbidities, were assigned to study treatment. Eighteen patients (72.0%) completed at least one cycle of treatment; however, one patient withdrew consent after two doses of cycle 2 and was not followed up for data collection. Seven (28.0%) subjects were withdrawn prior to completion of cycle 1 due to rapidly progressive disease and the need to address disease associated comorbidities. All 25 patients were assessed for toxicity. For tumor response (OS and PFS), there were two subsets (i) 24 patients who received at least one dose of EDV treatment and (ii) 17 patients

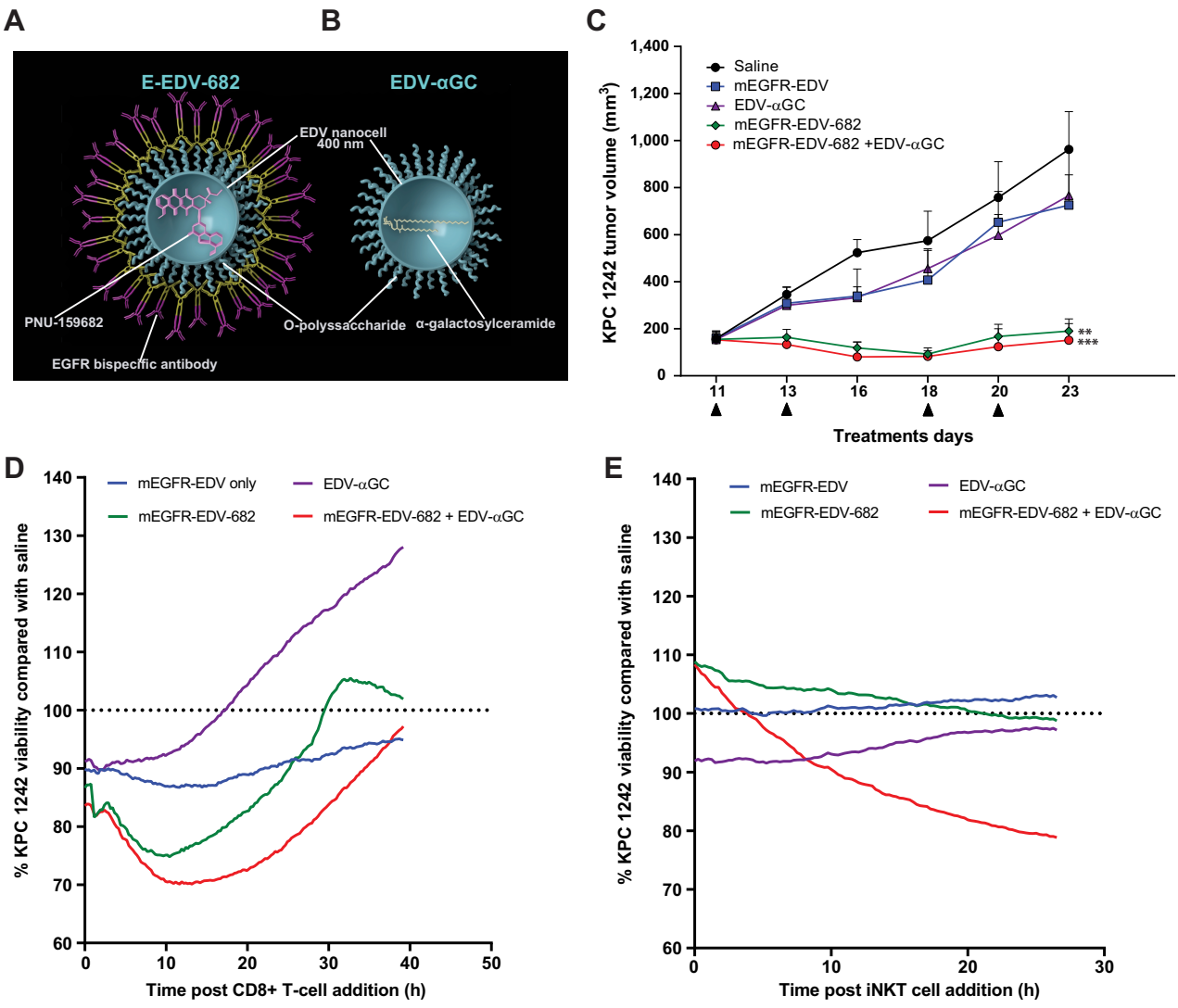

Figure 1.

Structure of E-EDV-D682/GC treatment and PDAC mouse model studies (**A**) EGFR-targeted, PNU-159682-packaged EDV and (**B**) α-galactosylceramide-packaged EDV. **C,** KPC 1242 mouse allograft tumor regression. ▲ = mice treated on days 11, 13, 18, and 20. mEGFR-EDV-682 and mEGFR-EDV-682+EDV-αGC compared with saline using ordinary one-way ANOVA; **, $P < 0.01$; ***, $P < 0.001$ (Prism v 9.5.0). **D,** Antitumor effects of the *ex vivo* CD8+ T cells and (**E**) iNKT cells coincubated with KPC 1242 cells and analyzed using xCELLigence.

[evaluable subset (ES)] who completed at least one cycle of EDV treatment. The seven patients who did not complete one cycle of treatment were also assessed for toxicity and OS, but PFS could not be measured because intracycle radiologic examination was not performed, and these patients all had rapid PD.

Baseline demographic and clinical characteristics of all 25 enrolled patients are shown in **Table 1**. Median age was 67 years (range, 47–82), 13 patients (52.0%) were male, and 12 (48.0%) were female. The weight ranged from 43 kg to 100 kg (median, 71.0 kg). All patients had ECOG performance status of ≤1. Eighteen patients (72.0%) were white, two Middle Eastern (8.0%), two Asian (8.0%), and three (12.0%) were not recorded.

Among the 25 treated patients, (i) seven (28.0%) had received Whipple surgery, (ii) one had received an aborted Whipple procedure with cholecystectomy, (iii) one, aborted distal pancreatectomy with splenectomy, (iv) 23 (92.0%) had received at least one line of chemotherapy, (v) three (12.0%) had received at least one course of radiotherapy, one on liver and the other two on pancreas, respectively (**Table 1**). Regarding chemotherapy, (i) 17 (68.0%) received at least one course of treatment with gemcitabine plus abraxane, (ii) 11 (44.0%) had received FOLFIRINOX, including leucovorin, 5-fluorouracil, irinotecan, and oxaliplatin. Prior therapies received by individual patients are shown in Supplementary Table S4.

Table 1. Baseline characteristics.

	PDAC ($N = 25$) n/N (%)
Age, y	
Median	67
Range	47–82
≥70	8 (32.0)
Sex	
Male	13 (52.0)
Female	12 (48.0)
Weight, kg	
Median	71.0
Range	43–100
Ethnicity	
White	18 (72.0)
Asian	2 (8.0)
Middle Eastern	2 (8.0)
Not reported	3 (12.0)
ECOG performance status score	
0	16 (64.0)
1	9 (36.0)
Tumor histology	
PDAC	25 (100.0)
EGFR expression on tumor metastasis	25 (100.0)
M1	14 (56.0)
M0	6 (24.0)
TNM staging not recorded[a]	5 (20.0)
Prior cancer treatment	
Surgery (resection at diagnosis)	7 (28.0)
Chemotherapy	
Nil	2 (8.0)
First line	23 (92.0)
Second line	11 (44.0)
Radiation therapy	3 (12.0)

Data are n/N (%) unless otherwise specified.
[a]TNM (tumor, nodes, and metastasis) staging was not recorded for four patients and metastasis status was not recorded for one patient.

Safety and tolerability

Nineteen of the 25 patients (76.0%) experienced at least one treatment-related AE (**Table 3**). However, all AE were mild or moderate, graded 1 to 2 in severity, and self-recovered within hours or recovered with remedial therapy.

Common treatment-related AE were general disorders and infusion-related reactions (chills, pyrexia, tremor, tachycardia, hypertension, hyperhidrosis) reported in 18 patients (72.0%), fatigue in six (24.0%), headache in three (12.0%), and lethargy in one (4.0%). The next most common treatment-related AE were musculoskeletal and connective tissue disorders, with back pain (grade 1–2 in severity) reported in eight patients (32.0%).

No patient experienced any grade 3, 4, or 5 treatment-related AE, and overall E-EDV-D682/GC was well tolerated, with no safety concerns in this patient population.

Dose reductions of either component of the treatment regimen (E-EDV-D682 or EDV-GC) or discontinuations attributed to study drug toxicity did not occur in any of the patients. There were no treatment-related deaths. The MTD was not identified, as no DLT were observed.

Response to therapy

Tumor response

Seven patients rapidly deteriorated prior to completion of cycle 1 and were withdrawn from the study to treat their comorbidities, and one patient withdrew his consent and was not followed up for OS. The remaining 24 patients were assessed for tumor response. Seventeen patients (68.0%, ES) completed cycle 1, and this subset was also assessed for tumor response. Among the ES, patients completed multiple cycles of EDV treatment (range, two to nine cycles) and eight patients (47.1%, overall, 28.6%) presented with stable disease (SD) at the completion of cycle 1 (**Table 4**).

The best response was a PR in regimen 2. Patient 2-CB12–1 showed SD at the completion of cycle 1 (**Table 4**); partial response at the completion of cycle 2 (iPR, iRECIST 2017), and continued with PR for a further three cycles of treatment before withdrawing at the end of cycle 6, due to unconfirmed progressive disease on radiologic examination (iUPD, iRECIST 2017). The patient's duration of response was 7.5 months (224 days), and OS was 19.7 months.

At the end of cycle 1, nine patients (53.0%) had iUPD. Two of these (**Table 4**; 1-CB14–1 and 1-CB47–1) were deemed by the treating oncologist to be deriving clinical benefit and continued subsequent cycle of treatment. Both patients completed two full cycles of treatment with an OS of 6.5 months (194 days) and 5 months (150 days) respectively. Similarly, patients 1-CB06–1 and 2-CB43–1 were graded iUPD on radiologic examination after completing two and four cycles, respectively. However, both patients appeared to show clinical improvement and maintained further treatment. Patient 1-CB06–1 completed four full cycles of treatment with an OS of 9.5 months (285 days), and 2-CB43–1 completed nine cycles with over 1-year OS (13.5 months, 406 days).

OS

The median OS (mOS) for subset 1 who received at least one dose of EDV (24 patients) was 4.4 months (131 days; range, 17–591 days; 95% CI, 4.1–8.1 months) and the Kaplan–Meier estimation is shown in **Fig. 2A**. However, in the ES (17 patients) where patients completed at least one cycle of treatment, the mOS was 6.9 months (208 days; range, 83–591 days; 95.0% CI, 5.6–10.3 months), while the mOS of the seven patients who were withdrawn prior to the

Table 2. Dosing regimens employed in Study ENG9.

Dosing Regimen	Maximum dose E-EDV-D682/ syringe	Maximum dose EDV-GC/syringe	Maximum dose total EDV E-EDV-D682/ GC/syringe
Regimen 1: 8-week schedule **Cycle 1:** • Biweekly dosing for 2 weeks then weekly dosing for 5 weeks • Week 8 as treatment free for radiologic evaluation **Subsequent cycles:** • Weekly dosing for 7 weeks at a maximum dose level attained in cycle 1 • Week 8 as treatment free for radiologic evaluation	5×10^9	5×10^8	5.5×10^9
Regimen 2: 8-week schedule **Cycle 1**: • Biweekly dosing for 2 weeks then weekly dosing for 5 weeks • Week 8 as treatment free for radiologic evaluation **Subsequent cycles:** • Weekly dosing for 7 weeks at a maximum dose level attained in cycle 1 • Week 8 as treatment free for radiologic evaluation	7×10^9	1×10^9	8×10^9
Regimen 3: 5-week schedule **Cycle 1**: • Combination of E-EDV-D682/GC followed by single-agent E-EDV-D682 given 30 min apart • Biweekly dosing for 2 weeks then weekly dosing for 2 weeks • Week 5 as treatment free for radiologic evaluation **Subsequent cycles:** • Combination of E-EDV-D682/GC followed by single-agent E-EDV-D682 given 30 min apart • Weekly dosing for 4 weeks at a maximum dose level attained in cycle 1 • Week 5 as treatment free for radiologic evaluation	7×10^9 – x 2 doses administered 30 min apart	*1×10^9 *(odd doses only 1, 3, 5 etc.)	8×10^9
Regimen 4: 5-week schedule **Cycle 1**: • Bolus injection of two doses of E-EDV-D682/GC given 45 min apart • Biweekly dosing for 2 weeks then weekly dosing for 2 weeks. • Week 5 as treatment free for radiologic evaluation **Subsequent cycles:** • Bolus injection of two doses of E-EDV-D682/GC given 45 min apart • Weekly dosing for 4 weeks at a maximum dose level attained in cycle 1 • Week 5 as treatment free for radiologic evaluation	7×10^9 x 2 doses administered 45 min apart	1×10^9 x 2 doses administered 45 min apart	8×10^9 x 2 doses administered 45 min apart
Regimen 5: 5-week schedule **Cycle 1**: • Bolus injection of three doses of E-EDV-D682/GC given 45 min apart • Biweekly dosing for 2 weeks then weekly dosing for 2 weeks • Week 5 as treatment free for radiologic evaluation **Subsequent cycles:** • Bolus injection of three doses of E-EDV-D682/GC given 45 min apart • Weekly dosing for 4 weeks at a maximum dose level attained in cycle 1 • Week 5 as treatment free for radiologic evaluation	7×10^9 x 3 doses administered 45 min apart	1×10^9 x 3 doses administered 45 min apart	8×10^9 x 3 doses administered 45 min apart

completion of cycle 1 was 1.8 months (54 days; range, 17–72 days; 95.0% CI, 1.2–2.2 months). OS of individual patients in the ES is listed in **Table 4**.

Among the ES of 17 patients, one did not receive any prior treatment. Six received one line of chemotherapy (subgroup 1), while 10 received more than one line of prior treatment, including chemotherapy and radiation treatment (subgroup 2). To examine whether prior treatment presents an impact on the OS of the ES with the IP treatment, the mOS of the two subgroups was analyzed. The mOS of subgroup 1 was 6.9 months (208 days; range, 83–591 days; 95.0% CI, 3.9–13.9 months) and the mOS of subgroup 2 was 7.0 months (211 days; range, 111–406 days; 95.0% CI, 5.1–9.3 months). The comparison between the two subgroups was not statistically significant ($P = 0.51$).

PFS

PFS was based on 22 progressions recorded with three patients being censored. The median PFS (mPFS) was 1.8 months (54 days; range, 14–364 days; 95.0% CI, 1.8–4.5 months) and the PFS Kaplan–Meier plot is shown in **Fig. 2B**. The mPFS of the ES was 2.2 months (65 days; range, 14–364 days; 95.0% CI, 2.5–5.8 months).

The two longest time to development of progressive disease were 12 months (364 days) and 10.3 months (310 days) in patient 2-CB12–1 on dose regimen 2 and patient 2-CB43–1 on dose regimen 4, who completed six and nine cycles of treatment, respectively. Five subjects completed a minimum of three full cycles and remained progression-free for 6.4, 7.6, 7.8, 10.3, and 12 months with an mPFS of 7.8 months (233 days; range, 193–364 days; 95.0% CI, 6.8–10.9 months).

Table 3. Treatment-related AE.

System organ class preferred term	Maximum severity grade 1–5 CTCAE V5.0	*N* = 25 n/N (%)
Any treatment-related AE (possibly, probably, and definitely related)		
Gastrointestinal disorders		
Nausea	1	4 (16.0)
Vomiting	1	4 (16.0)
Abdominal pain	1	1 (4.0)
Constipation	1	1 (4.0)
General disorders and administration site conditions		
Infusion-related reactions (chills, pyrexia, tremor, tachycardia, hypertension, and hyperhidrosis)	1–2	18 (72.0)
Fatigue	1–2	6 (24.0)
Headache	1–2	3 (12.0)
Pain	1	1 (4.0)
Lethargy	1	1 (4.0)
Peripheral edema	1	1 (4.0)
Metabolism and nutrition disorders		
Decreased appetite	1	3 (12.0)
Musculoskeletal and connective tissue disorders		
Back pain	1–2	8 (32.0)
Myalgia	2	1 (4.0)
Muscle spasms	1	1 (4.0)
Arthralgia	1	1 (4.0)
Nervous system disorders		
Dysgeusia	1	1 (4.0)
Confusional state	1	1 (4.0)
Disorientation	1	1 (4.0)
Skin and subcutaneous tissue disorders		
Pruritus	1	1 (4.0)
Rash, maculopapular	1	1 (4.0)
Investigations		
Elevated bilirubin	2	1 (4.0)
Elevated ALT	1	1 (4.0)
Elevated AST	1	1 (4.0)

Data are n/N (%) in the PDAC cohort. Grade 1–2 AE were reported. No treatment-related grade 3, grade 4 or 5 toxicity occurred.
Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase.

Body weight stabilization

Body weight was a prespecified assessment that took place at screening and prior to each EDV cycle. The results (**Fig. 2C**) show that three of the 17 patients in the ES had weight loss during the study treatment (i.e., loss of ≥ 5% of their pretreatment weight). The majority of patients [14 patients (82%)] did not experience weight loss and six patients showed a gain in weight over the duration of treatment (Supplementary Table S5). None of the patients had received any treatment for weight stabilization.

Inflammatory response and immunogenicity

Inflammatory cytokines in patient sera, (IL6, IL8, TNFα, and IFNγ) were measured by ELISA at each pre-EDV dose and 3 hours postdose and showed (**Fig. 2D** and **E**) an elevation at 3 hours postdose but returned to normal at each predose and remained within physiologic levels without any AE. Subsequent dosing did not result in augmented cytokine levels. The anti-inflammatory cytokine IL10 showed similar results with no AE. There were no significant differences between dosing regimens 2 to 5. Dosing regimen 1 had significantly lower levels of cytokines compared with all other regimens.

Supplementary Figure S1 shows the average titer of anti-LPS antibodies by dosing regimen. All subjects in general showed a rise in titer of anti-LPS IgG following the EDV treatment, and the titer reached a peak by the last dose of cycle 1 and were maintained at that level despite repeat dosing of subsequent cycles.

Representativeness of study participants

The representativeness of this study cohort is described in supplementary Supplementary Table S6.

Discussion

The prognosis for patients with PDAC remains dismal, likely due to the rapid development of chemoresistance, heterogeneous nature of pancreatic tumors, late-stage detection in greater than 80.0% of patients, a low opportunity for radical resection and postresection, systemic recurrence rates as high as 80% to 0.0% (26). Hence, when patients exhaust treatment options, the survival with best supportive care is at best a couple of months, and this is associated with rapid weight loss and poor quality of life.

Our earlier studies had shown that in patients with advanced PDAC, the tumor cells exhibit multiple drug resistance and hence all conventional anticancer drugs are ineffective (11). However, these tumor cells were highly sensitive to super-cytotoxic drugs like PNU-159682, and this drug was selected for packaging in the EDV nanocells. The toxicity of PNU-159682 precludes its use as a free drug (13).

This was the first clinical trial where PNU-159682 was administered intravenously in patients. Nine to 76 repeat doses of EGFR-EDV-D682/GC were delivered in the 17 patients with advanced PDAC, and the results showed minimal to no toxicity (**Table 3**). In previous studies, we had shown that once anticancer drugs are packaged in EDV, they do not leak *in vivo* and are only delivered intracellularly within tumor cells due to initial passive tumor targeting via the tumor associated leaky vasculature followed by active tumor cell targeting via engagement of EDV surface-attached bispecific antibody to the tumor cell surface receptor, macropinocytosis of the EDV, breakdown in lysosomes and intracellular drug release (9–11). The results in this trial show that a super-cytotoxic drug packaged in EDV can be safely administered systemically in human patients with cancer and can be used to overcome multidrug resistance in tumor cells found in patients with late-stage cancer.

Eight (47.0%) of the 17 patients presented SD at the completion of cycle 1. Because all the patients in this study presented with tumors that exhibited multiple drug resistance, they had already undergone all available treatment options and experienced treatment failure with previous chemotherapies. Consequently, the observed outcome suggests that PNU-159682 is likely to have been delivered to the PDAC cells in the patients who showed disease stabilization.

There are several known super-cytotoxic drugs like PNU-159682 (duocarmycin, mytansine, etc.) that are able to overcome multidrug resistance but have no therapeutic window due to extreme toxicity. This study is the first to demonstrate safe and effective delivery of such drugs in patients with advanced PDAC and releases the limitation on the arsenal of drugs that can be used to address multidrug resistance. Treatment of advanced PDAC has been limited to the use of very few drugs for more than 3 decades since the approval of gemcitabine in 1997, and each of these drugs rapidly loses antitumor efficacy due to multidrug resistance. An additional issue of note is that all the conventional chemotherapeutic drugs used as first- or second-line therapy in advanced PDAC are administered at a dose of between

Table 4. Tumor response.

Subject ID	Metastasis at diagnosis	Dosing regimen (R)	Cycles completed	Total doses	Tumor response	Duration of clinical benefit (d/mo)	OS (d/mo)	PFS (d/mo)
2-CB12–1	M1	R2	6	44	iSD/iPR	365/12.1	591/19.7	331/11.0
1-CB07–1	M1	R1	1	9	iUPD	57/1.9	508/16.9	57/1.9
2-CB43–1	M0	R4	9	76	iSD	310/10.3	406/13.5	135/4.5
1-CB05–1	MX	R1	2	20	iSD	136/4.5	333/11.1	136/4.5
1-CB06–1	M1	R1 & R2	R1: 2/R2: 2	30	iSD	233/7.8	285/9.5	108/3.6
1-CB45–1	TNM staging not recorded	R4 & R5	R4: 1/R5: 1	30	iSD	65/2.2	243/8.1	65/2.2
1-CB30–1	M1	R3 & R4	R3: 4/R4: 1	52	iSD	228/7.6	234/7.8	228/7.6
1-CB01–1	M0	R1	3	23	iSD	193/6.4	228/7.6	193/6.4
2-CB15–1	M1	R2	2	16	iSD	110/3.7	208/6.9	110/3.7
1-CB14–1	M1	R2	2	16	iUPD	107/3.6	194/6.5	49/1.6
1-CB16–1	M1	R2	1	9	iUPD	50/1.7	154/5.1	50/1.7
1-CB47–1	M0	R5	2	30	iUPD	62/2.1	150/5.0	30/1.0
2-CB22–1	TNM staging not recorded	R2	1	9	iUPD	49/1.6	111/3.7	49/1.6
2-CB32–1	M0	R3	1	12	iUPD	60/2.0	111/3.7	32/1.1
2-CB42–1	TNM staging not recorded	R4	1	12	iUPD	37/1.2	111/3.7	37/1.2
2-CB41–1	M1	R3	1	12	iUPD	30/1.0	84/2.8	30/1.0
1-CB46–1	M1	R4	1	12	iUPD	14/0.5	83/2.8	14/0.5

Abbreviations: R, regimen; iPR, immune partial response; iSD, immune stable disease; iUPD, immune unconfirmed progressive disease.

150,000 μg to 1.7 million μg per dose in an average 1.7 m^2 patient. In contrast, the maximum dose of PNU-159682 administered in 8×10^9 E-EDV-D682 was 3 μg. This trial is also the first demonstration of therapeutically effective drug doses to be as low as 3 μg. This is likely because the antibody-targeted EDV specifically deliver the packaged drug intracellularly in tumor cells, and each EDV nanocell carries approximately 350,000 molecules of PNU-159682.

A distinctive feature of PDAC is its extensive and dense desmoplastic stromal tumor microenvironment that is thought to contribute to treatment resistance (27) and hinders delivery of chemotherapy drugs (28–30). Therefore, it is surprising that greater than 40.0% of the patients showed tumor stabilization suggesting that the EDV were likely able to access the PDAC cells despite the stroma. In further clinical trials, it would be interesting to carry out imaging/biopsy studies to determine if the EDV and the packaged drug are found in PDAC cells. We have previously carried out such studies in a canine clinical trial on 17 dogs with stage IV glioblastoma where ^{123}I-labeled EDV administered intravenously were detected (SPECT/MRI imaging) in 3 hours to accumulate in the glioblastoma tumor tissue (31).

Nine patients (53.0%) had unconfirmed progressive disease after completing cycle 1 treatment. However, the treating oncologists observed that two of these patients (1-CB14–1 and 1-CB47–1) were deriving clinical benefit and were put on further treatment cycles. The same occurred in two other patients 1-CB06–1 and 2-CB43–1 when they completed two and four cycles, respectively. On this basis, the four patients showed an average of OS of 256 days (8.6 months) from the start of the EDV treatment. This OS exceeded expectations for patients with advanced PDAC who had exhausted treatment options.

This phenomenon has been observed with immuno-oncology drugs including ipilimumab and nivolumab where response was atypical with unique patterns compared with conventional cytotoxic drugs (32, 33). An initial radiologic progression and delayed tumor shrinkage was termed as pseudoprogression (34). The use of RECIST in this situation resulted in premature discontinuation of therapy, although there was a later response, and these delayed but durable responses were associated with prolonged survival (33).

Patients with advanced metastatic PDAC who have no treatment options left have a life expectancy of only 8 to 16 weeks (35–37), an observation further borne out in this study where the seven patients who were withdrawn prior to the completion of cycle 1, the mOS was 1.8 months (54 days; range, 17–72 days; 95.0% CI, 1.2–2.2 months). Interestingly, the mOS of the 17 evaluable patients was 6.9 months (208 days; range, 83–591 days; 95.0% CI, 5.6–10.3 months) and ranged from 5 to 19.7 months. Five (29.0%) of these 17 patients showed OS of 8.1, 9.5, 11.1, 13.5, 16.9, and 19.7 months.

PDAC is also characterized by weight loss (WL) (38) due to anorexia, malabsorption, and cancer cachexia (39); at presentation, most patients with PDAC show clinically significant WL (40). Cachexia is a multifactorial syndrome characterized by progressive involuntary WL, loss of skeletal muscle mass, and systemic inflammation (41, 42), and WL is associated with a poor prognosis, postop infections, and decreased therapeutic efficacy (39, 43–47). A recent clinical study ($n = 92$; ref. 48) in patients with advanced PDAC that was randomized and with a control arm reported that cachexia was diagnosed in 41.4% of all patients, and a high percentage (49.2%) of the patients reported recent weight loss. In two separate studies (49, 50) involving patients with nonadvanced pancreatic cancer ($n = 82$ and 202, respectively), a significant number of patients experienced weight loss—62% in the first study and 46% in the second study.

The data in this trial showed weight stabilization in 14 of the 17 evaluable patients with end-stage PDAC (82.0%; **Fig. 1C**) during the duration of the EDV treatment. These data while observed in only a small number of patients suggest that the EDV treatment likely triggers neutralization of critical weight loss molecular pathway(s) and thereby stabilizes the weight of such end-stage patients. Further studies will be required in larger clinical trials to determine if this is an anecdotal observation or a consistent phenomenon because currently there is no treatment available for WL in patients with end-stage cancer.

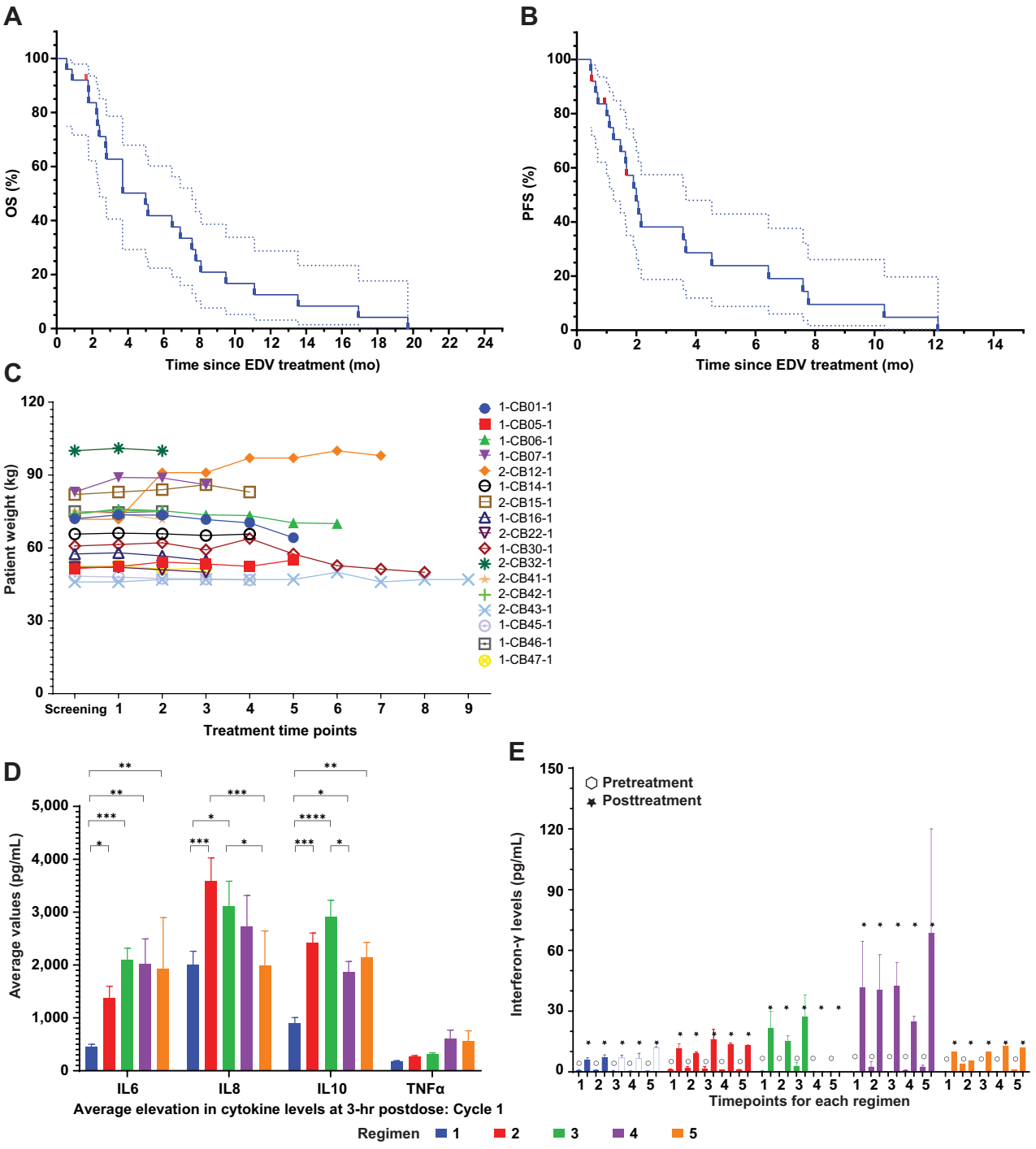

Figure 2.

Trial participant survival data, weight data, and cytokine response (**A**) Kaplan–Meier plot of OS. The point highlighted in red indicates the patient withdrew his consent-censored). mOS: 4.4 months (based on 24 deaths, 131 days; range, 17–591 days); 95% CI, 4.1–8.1 months. OS rate for 25 patients at 6 and 12 months was 40.0% and 12.0%, respectively. **B,** Kaplan–Meier plot of PFS. The points highlighted in red indicate the censored data, including one patient withdrew his consent, two other patients were withdrawn from the study due to their serious comorbidity. mPFS: 1.8 months (based on 22 patients, 54 days; range, 14–364 days); 95% CI, 1.8–4.5 months. **C,** Weight data taken at timepoints before dose treatment. **D,** Cytokine response for IL6, IL8, IL10, and TNFα at 3-hour post-dose during cycle 1 across five dosing regimens; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$ (Prism v 9.5.0, 2-way ANOVA). **E,** IFNγ response. The x-axis shows five timepoints of measurement for each regimen during the study, predose, and 3-hour postdose.

Authors' Disclosures

S. Pattison reports other support from EnGeneIC outside the submitted work. N.B. Amaro-Mugridge reports other support from EnGeneIC outside the submitted work. B.R.G. Williams reports grants from EngeneIC Pty Ltd. during the conduct of the study and is director and shareholder of EngeneIC Pty Ltd. J.A. MacDiarmid reports other support from EnGeneIC outside the submitted work. H. Brahmbhatt reports other support from EnGeneIC Pty Ltd. outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

V. Ganju: Conceptualization, resources, formal analysis, supervision, validation, investigation, methodology, writing–original draft. **G. Marx:** Conceptualization, supervision, validation, methodology, writing–review and editing. **S. Pattison:** Resources, formal analysis, supervision, validation, investigation, project administration. **N.B. Amaro-Mugridge:** Resources, investigation, visualization, writing–review and editing. **J.-T. Zhao:** Formal analysis, validation, visualization, writing–review and editing. **B.R.G. Williams:** Conceptualization, methodology, writing–original draft, writing–review and editing. **J. A. MacDiarmid:** Conceptualization, resources, supervision, funding acquisition, investigation, visualization, methodology, writing–original draft, writing–review and editing. **H. Brahmbhatt:** Conceptualization, resources, supervision, funding acquisition, investigation, visualization, methodology, writing–original draft, writing–review and editing.

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Note

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