Preclinical Characterization of HPN536, a Trispecific, T Cell-activating Protein Construct for the Treatment of Mesothelin-Expressing Solid Tumors

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**Translational Relevance:** Patients with mesothelin (MSLN) overexpressing tumors, including ovarian, pancreatic, lung and triple-negative breast cancer, have a high unmet clinical need. A number of MSLN-targeted therapeutics have been developed that show limited efficacy and safety in clinical trials. HPN536 is a novel MSLN-targeted trispecific T cell-activating protein construct that can potently redirect T cells to lyse tumor cells and was remarkably well tolerated in non-human primates at single doses up to 10 mg/kg, which is far above the expected therapeutic dose level. Our findings suggest that HPN536 has the potential for high clinical activity and a wide therapeutic window. Its long serum half-life supports once weekly dosing in humans. Currently, HPN536 is the only MSLN-targeting, T cell-engaging biological in clinical testing.
ABSTRACT

Purpose: Mesothelin (MSLN) is a GPI-linked tumor antigen overexpressed in a variety of malignancies, including ovarian, pancreatic, lung and triple-negative breast cancer. Early signs of clinical efficacy with MSLN-targeting agents have validated MSLN as a promising target for therapeutic intervention, but therapies with improved efficacy are still needed to address the significant, unmet medical need posed by MSLN-expressing cancers.

Experimental Design: We designed HPN536, a 53-kDa, trispecific, T cell-activating protein-based construct (TriTAC), which binds to MSLN-expressing tumor cells, CD3ε on T cells and to serum albumin. Experiments were conducted to assess the potency, activity and half-life of HPN536 in in vitro assays, rodent models and in non-human primates.

Results: HPN536 binds to MSLN-expressing tumor cells and to CD3ε on T cells leading to T cell activation and potent redirected target cell lysis. A third domain of HPN536 binds to serum albumin for extension of plasma half-life. In cynomolgus monkeys, HPN536 at doses ranging from 0.1 to 10 mg/kg demonstrated MSLN-dependent pharmacologic activity, was well tolerated and showed pharmacokinetics in support of weekly dosing in humans.

Conclusions: HPN536 is potent, well tolerated and exhibits extended half-life in non-human primates. It is current in phase 1 clinical testing in patients with MSLN-expressing malignancies (NCT03872206).
INTRODUCTION

Redirection of cytotoxic T cells with bispecific antibody constructs for cancer therapy has been validated in the clinic (1-6). Blinatumomab is the first and thus far only bispecific T-cell engager approved by the FDA (7). T cell-engaging biologicals function by forming an immunological cytolytic synapse between cancer target cells and T cells, which leads to target cell lysis independent of T cell receptor specificity, peptide antigen presentation by HLA, and T cell costimulation. Despite the clinical success of blinatumomab for treating relapsed and refractory acute lymphoblastic leukemia (ALL), other molecules including BiTE antibodies showed only limited activity in the treatment of solid tumors (8,9). Their short plasma half-life required continuous intravenous infusion limiting their utility for most solid tumor indications. Novel designs for T cell-engaging antibodies aim at overcoming limitations of the first generation and are already being tested in clinical trials (10).

The Tri-specific T Cell-Activating Construct (TriTAC) design has been specifically developed to treat solid tumors (11). TriTACs consist of a single polypeptide chain aligning three humanized, antibody-derived binding domains: a single domain antibody (sdAb) specific for a tumor antigen, a sdAb specific for serum albumin for half-life extension, and a single chain Fv (scFv) specific for the CD3ε subunit of the T cell receptor (TCR) complex (11). Their molecular size of 53 kDa is about one-third of that of an IgG. Binding of TriTACs to tumor antigen and CD3ε is monovalent, which minimizes off-target CD3ε clustering that can potentially lead to non-specific T cell activation. The absence of an Fc-gamma domain for half-life extension is functionally compensated by an albumin-binding domain. With HPN424 (11) and HPN536, the
first two TriTAC constructs are in phase 1 clinical testing in hormone-refractory prostate cancer and MSLN-overexpressing solid tumors, respectively.

Human mesothelin (MSLN) is produced as a 71-kDa precursor of 628 amino acids, which is expressed as a glycophosphatidylinositol (GPI)-linked cell-surface glycoprotein. Its 31-kDa N-terminal domain is released as a soluble protein, termed the megakaryocyte potentiating factor (MPF), while the 40 kDa C-terminal domain remains attached to the plasma membrane as mature MSLN (12-14). MSLN expression on normal tissue is confined to the single-cell mesothelial layer covering the surface of tissues and organs of the pleural, pericardial, and peritoneal cavities (13,15). MUC16/CA125 is a binding partner for MSLN, implicating a role for MSLN in cell adhesion (16,17). However, the precise physiological functions of MSLN have not been defined, and MSLN knockout mice exhibit no detectable phenotype or developmental abnormality (18).

MSLN is overexpressed in many malignancies including ovarian cancer (13,15,19), pancreatic cancer (20,21), non-small cell lung cancer (22-25)(23–26), triple negative breast cancer (26,27), and mesothelioma (28,29). In triple negative breast cancer (25) and in lung and pancreatic adenocarcinomas (22,23,30), overexpression of MSLN correlates with poor prognosis. Differential expression of MSLN in cancer versus normal tissue has made it an attractive target for MSLN-directed imaging agents and therapeutics (10,31-33). A challenge in developing MSLN-directed therapeutics is the expression of MSLN on normal mesothelial cells, potentially leading to dose limiting toxicities.

HPN536 specifically redirects T cells for potent redirected lysis of MSLN-expressing cancer cells with concomitant T cell activation. In three different mouse xenograft models,
HPN536 induced durable anti-tumor activity at very low doses. In cynomolgus monkeys, HPN536 was well tolerated, showed a long serum half-life, and elicited signs of target engagement on mesothelial structures.

MATERIALS AND METHODS

Protein production

Sequences of TriTACs, single domain antibodies, and extracellular domains of target proteins fused to an Fc domain or a hexahistidine tag were cloned into mammalian expression vector pcDNA 3.4 (Invitrogen) preceded by a leader sequence. Expi293 cells (Life Technologies) were maintained in suspension in Optimum Growth Flasks (Thomson) between 0.2 to 8 x 10^6 cells/ml in Expi293 media. Purified plasmid DNA was transfected into Expi293 cells in accordance with Expi293 Expression System Kit (Life Technologies) protocols and cultured for 4-6 days post transfection. Alternatively, HPN536 was produced in CHO-DG44 DHFR deficient cells (34). The amount of expressed proteins in conditioned media was quantitated using an Octet RED96 instrument with Protein A tips (ForteBio / Pall) using appropriate purified control proteins for a standard curve. Conditioned media from either host cell was filtered and purified by protein A affinity and desalted or subjected to preparative size exclusion chromatography (SEC) using an AKTA Pure chromatography system (GE Healthcare). Protein A purified TriTAC proteins were further purified by ion exchange and formulated in a buffered solution containing excipients. Final purity was assessed by SDS-PAGE by resolving 2.5 μg/lane on TRIS-Glycine gels and visualized with Simply Blue stain (Life Technologies). Native purity and was also assessed by
analytical SEC using a Yarra SEC150 3μm 4.6 x 150mm column (Phenomenex) resolved in an aqueous/organic mobile phase buffered at neutral pH on a 1290 LC system and peaks integrated with OpenLab Chemstation software (Agilent).

**In vitro affinity measurements**

Affinities of HPN536 analyte for albumin, CD3ε and MSLN ligands were measured by biolayer interferometry using an Octet RED96 instrument with streptavidin tips (ForteBio / Pall). Experiments were performed at 27°C in PBS plus casein in the absence or presence of 15 mg/ml HSA as described in Results and figure legends. Binding sensograms generated from empirically determined ligand loads, appropriate serial dilutions of known analyte concentrations, and association and dissociation times were then fit globally to a one-to-one binding model using Octet DataAnalysis 9.0 software.

**In vitro T cell dependent cell cytotoxicity (TDCC) and T cell activation assays**

T cells from healthy donors were purified from leukopaks (leukapheresis samples, STEMCELL Technologies) using EasySep™ Human T Cell Isolation Kits (STEMCELL Technologies 17951) following the manufacturer’s instructions. All cancer cell lines were obtained from ATCC with the exception of OVCAR8 cells which were obtained from the National Cancer Institute. Cell lines were passaged a maximum of 36 times after being received from ATCC. Cell line authentication and Mycoplasma testing was not performed. TDCC assays were performed as previously described (35). Briefly, luciferase expressing target cells and purified human T cells were seeded per well of a 384-well plate at a 10:1 T cell to target cell ratio. Target cell killing was assessed following incubation for 48 hours at 37°C and 5% CO2. Target cell viability was assessed by incubation with the SteadyGlo reagent (Promega). Luminescence was measured
using a Perkin Elmer EnVision® detection system. Activated T cells were identified by CD69 and CD25 surface expression (BD Biosciences) Samples were analyzed on a FACSelesta (BD Biosciences). Flow cytometry data were processed using FlowJo v10 software (FlowJo, LLC, Ashland, Oregon).

**Binding of HPN536 on MSLN-expressing OVCAR and T cells**

Cultured cells were incubated with 1 µg/mL HPN536 or anti-GFP TriTAC (control) for 1 hour. Binding was detected using Alexa647-anti-TriTAC antibody using a FACSelesta flow cytometer (BD Biosciences). The QIFIKIT (Dako) was used according to manufacturer’s instructions to estimate the number of MSLN molecules expressed per cell.

**Cytokines in the presence of T cells**

To measure the cytokines AlphaLISA kits were used (Perkin-Elmer) per the manufacturer’s instructions except that the assays were performed in 384 well plates instead 96 well plates. Plates containing conditioned media from TDCC assays were used for analysis. Plates were read on a Perkin-Elmer EnVision plate reader equipped with an AlphaLISA module.

**In vivo mouse efficacy studies**

All mouse studies were performed in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC) at Harpoon Therapeutics and Charles River. For TOV21G and HPAFII experiments, NCG (NOD-Prkdcزم26Cd52/Il2rgزم26Cd22/NjuCrl) mice received subcutaneous co-implants of human cancer cells (5 x 10^6) and human T cells (5 x 10^6) in 50% Matrigel (BD Biosciences) on day 0. Human T cells were expanded before implantation using human T Cell Activation/Expansion Kit (Miltenyi Biotec) according to manufacturer’s instructions. Mice were
dosed on days 1-15 (HPAFII Fig 4a and TOV21G Fig 4c) or days 7-16 (HPAFII Fig 4b) via intraperitoneal injection. For NCI-H292 experiments, NCG mice received subcutaneous co-implants of human cancer cells (1 x 10⁷) and human PBMCs (1 x 10⁷). Mice were administered HPN536 daily for 10 days starting on Day 6 days via intravenous injection. Tumor size was measured twice weekly and calculated using the following formula: tumor volume (mm³)=(w² x l)/2. Percent tumor growth inhibition (%TGI) was defined as the difference between the mean tumor volume (MTV) of the control group and the MTV of the treated group, expressed as a percentage of the MTV of the control group.

**Exploratory cynomolgus monkey dose range finding study**

The pharmacology, pharmacokinetics and toxicity of HPN536 was evaluated after a single intravenous bolus dose of 0.1, 1.0 or 10 mg/kg HPN536 in 1 male and 1 female cynomolgus monkey per group followed by either a 1- or 3-week post dose recovery period. The study followed the protocol and SOP’s of the testing facility (Charles River Laboratories) and was approved by their IACUC. Pharmacologic activity was evaluated by clinical observations, cytokine assessments, flow cytometry and evidence of target engagement by histology. Two research electrochemiluminescence assays, a functional assay and an anti-idiotype assay, were used for measuring HPN536 levels in serum. For the functional assay, HPN536 was captured with biotinylated CD3ε and was detected with a sulfo-tagged mesothelin (MSLN). For the anti-idiotype assay, HPN536 was captured with an anti-idiotype antibody recognizing the anti-albumin domain and was detected with a sulfo tagged CD3ε. Toxicokinetic parameters were estimated using Phoenix WinNonlin pharmacokinetic software. A non-compartmental approach consistent with the intravenous bolus route of administration was used for parameter
estimation. Toxicity endpoints included, daily morbidity and mortality, daily clinical observations, weekly body weights, daily food consumption, clinical pathology (hematology, clinical chemistry, coagulation), and anatomic pathology (gross necropsy, organ weights, histopathology).

RESULTS

Production, structure and biochemical characteristics of HPN536

Recombinant HPN536 has a molecular weight of approximately 53 kDa. A humanized llama sdAb specific for human MSLN is placed at its N-terminus (Fig. 1A). A humanized llama sdAb specific for human serum albumin (HSA) is placed in the middle of the molecule. The C-terminal end contains a humanized single-chain Fragment variable (scFv) specific for the human CD3ε subunit of the T cell receptor complex (TCR). GGGSGGGGS linkers connect the three binding domains.

HPN536 is produced by eukaryotic cell culture and secreted as a single, non-glycosylated polypeptide. Stability studies subjecting HPN536 to various stress conditions, including multiple freeze thaw cycles and storage at 4°C and 40°C for two weeks, suggest the protein is stable and stress resistant (Fig. S1). The high stability of HPN536 ensures limited aggregation, which would otherwise lead to CD3 clustering and non-specific T cell activation in the absence of target cells, and potential off-target side effects.

Biolayer interferometry analysis demonstrated that HPN536 binds with high affinity to recombinant or purified human and cynomolgus monkey MSLN, CD3ε, and albumin in the range of 0.21 to 6.7 nM (Fig. 1B). The $K_D$ values for binding to human and cynomolgus orthologs were found to be within 6-fold of each other for all three target antigens. HPN536 bound to mouse
MSLN and albumin with equilibrium binding constants of 210 nM and 170 nM, respectively, but did not detectably bind mouse CD3ε (Fig. 1B). As demonstrated by flow cytometry analysis, HPN536 exhibited surface binding to human MSLN-expressing OVCAR cells and purified human T cells, confirming that HPN536 recognized native MSLN and CD3ε expressed on cells (Fig. 1C).

Redirected tumor cell lysis by HPN536 in cocultures

The in vitro potency of HPN536 was evaluated in a T cell-dependent cell cytotoxicity (TDCC) assay. Primary resting human T cells were combined with target cells at a ratio of 10:1, incubated for 48 hours, and the viability of target cells determined. In co-cultures of MSLN-expressing OVCAR3 ovarian cancer cells and resting T cells from 5 different donors, HPN536 efficiently directed T cells to lyse OVCAR3 target cells with EC\textsubscript{50} values ranging from 1.3 to 2.5 pM (Fig. 2A and Tab. S1). No lysis of OVCAR3 cells was observed with a control TriTAC specific for green fluorescent protein (GFP) showing that sole binding to CD3ε on T cells was not sufficient to mediate cytotoxicity. HPN536 also redirected T cells for lysis of other ovarian cancer cell lines expressing MSLN, including Caov3, Coav4 and OVCAR8, but it was inactive against MSLN-negative cell lines MDAPCa2b and NCI-510A, demonstrating strict specificity for MSLN (Fig. 2B and Tab. S1). Because HPN536 contains an anti-albumin binder for half-life extension, TDCC was conducted in the presence of physiological levels of serum albumin. Serum albumin did not inhibit the ability of HPN536 to redirect human T cells to kill MSLN-expressing target cells and had a minimal impact on potency (Fig. S2).

Lysis by HPN536-engaged T cells was further explored with MSLN-expressing tumor cell lines of different histological origin, including three pancreatic (Hs766T, Capan-2, HPAFII) (Fig. 2C and Tab. 1), three non-small cell lung cancer (NCI-H596, NCI-H292, NCI-H1563) (Fig. 2D and
Tab. 1), and two mesothelioma cell lines (NCI-H2052 and NCI-H2452) (Tab. 1). EC$_{50}$ values of HPN536 for redirected lysis ranged from 2.3 to 15 pM across the various cell lines. HEK 293 cells genetically engineered to express either human or cynomolgus MSLN were killed with EC$_{50}$ values of 0.9 and 0.7 pM, respectively. PBMCs from Cynomolgus monkeys were also able to kill MSLN-expressing tumor cells in the presence of HPN536 (Fig. S3). These data support the use of cynomolgus monkey as a relevant species for toxicology studies with HPN536. Table 1 summarizes the EC$_{50}$ values across all cell lines of different histopathological origin as well as an estimate of the number of MSLN molecules expressed per cell as determined by flow cytometry.

**T cell activation by HPN536**

T cell activation by HPN536 was first assessed by induction of CD69 and CD25 surface expression on T cells. T cells from four normal donors were co-cultured with the MSLN-positive cancer cell line OVCAR8 at various concentrations of HPN536. Within 48 hours, HPN536 mediated a dose-dependent increase in the percentage of CD69- and CD25-positive T cells in co-culture with MSLN-positive OVCAR8 cells (Figs. 3A and B). A GFP-specific TriTAC control had no effect on new CD69 or CD25 expression on T cells. Depending on the T cell donor, between 5 and 20% of the T cells in culture upregulated CD69 and CD25 surface expression. Similar effects on T cell activation were observed in coculture with MSLN-positive tumor cell line Caov3 (Fig. S4). The EC$_{50}$ values of HPN536 for upregulation of CD69 and CD25 in the presence of MSLN-positive cancer cells ranged from 0.14 to 9.0 pM (Tab. S2).

T cell activation was also assessed by the release of inflammatory cytokines by T cells that were co-cultured with MSLN-expressing OVCAR8 cancer cells. As shown in Figures 3C and
3D, HPN536 mediated a dose-dependent secretion of TNFα and IFNγ into co-culture media. A GFP-specific control TriTAC did not cause cytokine release by T cells. Comparable results were observed with Coav3, Caov4 and OVCAR3 as target cells and with multiple T cell donors (Tabs. S3 and S4). Of note, induction of CD69, CD25 and release of cytokines by T cells was not observed in coculture with MSLN-negative tumor cell line MDAPCa2b (Fig. S5).

**Anti-tumor activity of HPN536 in mouse xenograft models**

The anti-tumor activity of HPN536 was assessed in three xenograft mouse models established from cancer types that express MSLN: HPAFII (pancreatic cancer), TOV21G (ovarian cancer), and NCI-H292 (non-small cell lung cancer). A daily dosing regimen was selected based on the reported half-life for mouse serum albumin of approximately 1 day (36,37). Moreover, HPN536 bound mouse serum albumin with a lower affinity than human serum albumin (K_D values of 170 nM versus 6.3 nM, respectively). Consistent with this, the serum half-life of HPN536 in mice was determined to be 24 hours (Fig. S6 and Tab. S5). In a first study, HPAFII tumor cells were subcutaneously co-implanted with T cells from normal human donors at an effector to target cell ratio (E:T) of 1:1 (Fig. 4A). Daily doses of HPN536 of 100, 20, or 4 μg/kg, were administered over 15 days by intraperitoneal (i.p.) injection starting one day after implantation. Doses of 20 μg/kg and 100 μg/kg HPN536 caused HPAFII tumor eradication, while 4 μg/kg only slightly delayed tumor outgrowth (Fig. 4A) compared to a GFP-specific control TriTAC at 100 μg/kg. In a second experiment, HPAFII cancer cells and T cells from a healthy donor at an E:T of 1:1 were co-implanted under the skin and tumors allowed to establish for 7 days until they reached an average volume of 170 mm^3 before a 10-day treatment with HPN536 was initiated. On day 24
post tumor implantation, both 500 and 100 μg/kg doses of HPN536 led to eradication of the established HPAFII tumors in mice (Fig. 4B).

For the TOV21G tumor model, mice were treated with 500, 100, and 20 μg/kg HPN536 one day following tumor co-implantation with human T cells. On day 35 post implantation, 500, 100, and 20 μg/kg HPN536 treatment groups showed TGI of 65.1%, 68.6%, 52.3%, respectively, and delayed tumor outgrowth with high statistical significance (Fig. 4C). For the NCI-H292 model, tumor cells were co-implanted with human peripheral blood mononuclear cells (PBMCs) and tumors allowed to grow for 6 days at which point the tumor volume reached about 27 mm$^3$ before treatment was initiated. On day 6, HPN536 was administered at daily doses of 500, 100, and 20 μg/kg for 10 days. A highly significant tumor inhibition was seen in both the 100 μg/kg and 500 μg/kg HPN536-treated groups compared to the vehicle-treated group (70.9% and 77.1% TGI, respectively)(Fig. 4D). These results demonstrate activity of HPN536 in controlling growth of MSLN-expressing tumors derived from different histological origins.
Pharmacokinetics of HPN536 in cynomolgus monkeys

Based on the species cross reactivity of HPN536 to cynomolgus MSLN, CD3, and albumin (see Fig. 1), the pharmacokinetics (PK) of a single dose of 0.1, 1 or 10 mg/kg was evaluated in cynomolgus monkeys (2 males and 2 females per dose group). An anti-idiotype assay (anti-anti-albumin used for capture and anti-anti-CD3 for detection) and a functional assay (recombinant CD3 and MSLN proteins used for capture and detection, respectively) were used for detection of HPN536. The serum concentration-time profile for HPN536 exhibited a biphasic decline for the dose range 0.1 to 10 mg/kg over the time course of the study (Fig. 5A). Non compartmental analysis of HPN536 exhibited a dose proportional increase in maximum serum concentration ($C_{\text{max}}$) and area under the concentration–time curve ($\text{AUC}_{0-\text{inf}}$). In addition, the volume of distribution at steady state ($V_{ss}$) was independent of dose. The clearance rate was in the range of 0.57 to 1.39 ml/h/kg and the mean terminal half-life ($T_{1/2}$) was between 49.0 to 113 hours, supporting the hypothesis that the human serum albumin binding domain engaged its target HSA to extend the serum half-life of HPN536 (Tab. S6). The time-concentration profiles determined with the two different assays overlaid (Fig. 5A), suggesting that HPN536 had retained its structural and functional integrity in cynomolgus monkeys over the course of the 3-week study. To examine in vivo stability and biological activity, HPN536-containing serum samples collected after 168 hours post HPN536 administration were tested for redirected tumor cell lysis in the TDCC assay. As shown in Figure 5B, HPN536 contained in the 168-hour serum samples from monkeys were as potent in the TDCC assay as the HPN536 reference stored at -80°C.

Pharmacodynamics and toxicology of HPN536 in cynomolgus monkeys
Single doses of HPN536 were well-tolerated in non-human primates (NHPs) up to 10 mg/kg and there were no dose-limiting toxicities observed. HPN536 administration resulted in transient, non-adverse changes in clinical pathology parameters (Figs. 5C-E, Tab. S7) and cytokines. Consistent with T cell activation by HPN536, transient mild to moderate decreases in circulating lymphocytes were observed between days 1 and 2 post drug administration that trended back to control or baseline values by days 3 or 8 (Tab. S7). They included helper T lymphocytes, cytotoxic T lymphocytes, natural-killer cells, and B lymphocytes as observed by flow cytometry. Figure 5C exemplifies a transient reduction in T lymphocytes that recovered by 168 hours post drug administration. A small fraction of T cells showed upregulation of T cell activation marker CD69 in response to HPN536 that was not seen in vehicle-treated animals (Fig. 5D). T cell activation in response to HPN536 was also evident from a dose-dependent increase in the inflammatory cytokine IL-6 in serum (Fig. 5E). At 10 mg/kg, IL-6 levels peaked between 4- and 8-hours post HPN536 administration and had declined by 24 hours post-dose. Increases in serum levels of IFNγ and IL-2 were also observed between 4- and 8-hours post-dose in a subset of animals treated at 10 mg/kg (Fig. S7). No consistent changes in serum levels were observed for other cytokines, including IL-4, IL-5, IL-10 and TNFα (Fig. S7).

Single doses of HPN536 did not elicit gross pathologic changes or dose-limiting toxicities. The main histopathologic finding was a moderate, dose-dependent mesothelial hypertrophy accompanied by a mixed immune cell infiltration and extracellular matrix deposition. This effect was most pronounced in animals treated at the highest dose level of 10 mg/kg HPN536. Figure 6 compares a microscopic section through the pulmonary mesothelial layer of an animal receiving a 10 mg/kg dose (middle panel) with a section from a vehicle-
treated animal (left panel). These HPN536-related mesothelial changes were observed in fewer tissues and at a lower incidence and/or severity 3 weeks post dosing when compared to findings at one-week post dosing, possibly reflecting reversibility of the histopathological effect (Fig. 6, middle and right panels).

**DISCUSSION**

Multiple MSLN-targeted therapies for the treatment of MSLN-expressing malignancies have entered clinical development in recent years (38,39). These include the antibody amatuximab (40), MSLN-based vaccines (41,42), CAR-T cells (32), immunotoxins (33), and antibody drug conjugates (43). Some clinical benefit has been reported for the immunotoxin SS1P (33,44,45) and the antibody-drug conjugate DMOT4039A (46). Likewise, MSLN-specific CAR-T cells have shown some benefit after intraperitoneal administration to patients with mesothelioma in combination with an anti-PD1 antibody (32,47,48). Challenges of current MSLN-targeted therapies are low response rates and narrow therapeutic windows. To overcome these, we have generated HPN536, which currently is the only T cell-engaging antibody construct in clinical testing in patients with MSLN-expressing cancers.

While many T cell engager formats in development rely on an Fc-gamma domain for half-life extension, HPN536 utilizes a sdAb binding to HSA. Here we show that the sdAb can confer a prolonged serum half-life of the 53-kDa TriTAC for up to 113 hours in non-human primates. By contrast, the 55-kDa BiTE antibody blinatumomab had a serum half-life in patients of only two hours and therefore required continuous IV infusion (7,49). A weekly or perhaps less frequent dosing schedule may be sufficient to maintain appropriate plasma drug
concentration of HPN536 for anti-tumor activity in the clinic, which would overcome a limitation of the canonical BiTE format. Of note, the in vitro biological activities of HP536 were only slightly affected by the presence of physiological concentrations of serum albumin, which seems to be owed to positioning of the albumin-binding sdAb in the middle of the TriTAC molecule.

Two single domain antibodies were used to engineer HPN536 in order to achieve high protein stability and to minimize the overall size of the TriTAC. Single domain antibodies were shown to have a higher stability than conventional antibodies and derived fragments that require pairing of heavy and light chain variable domains (50,51). This may explain the high stability of HPN536 in the circulation of cynomolgus monkeys for up to 7 days, where the TriTAC retained virtually the same biological activity as stored reference material. The inherent stability of sdAbs is retained with alignment of multiple domains on a single polypeptide chain as exhibited by low aggregation propensity. Notably, HPN536 remained monomeric, i.e., monovalent, even after incubation for up to 14 days at 40°C (Fig. S1). We consider this to be important for HPN536 for reduction of non-specific T cell activation and avoidance of off-target T cell activation in the periphery as can be caused by formation of aggregates, which often is a problem with multivalent anti-CD3 moieties. Finally, the use of a small albumin-binding sdAb for half-life extension in lieu of a Fc-gamma domain allowed reduction of the overall molecular size of TriTACs to a third of an IgG molecule. On theoretical grounds, we hypothesize that the small size and globular shape of HPN536 may improve diffusion into and across tumor tissue (52) for better T cell engagement and anti-tumor activity. Although HPN536 binds albumin and possibly soluble MSLN, these interactions are noncovalent. As a result, a small fraction of
HPN536 could remain unbound at any given time that can potentially diffuse as a ~53 kDa protein and more readily access the tumor microenvironment.

Redirected lysis and T cell activation by HPN536 was dependent on binding of the TriTAC molecule to MSLN expressed on cancer cells and to CD3ε expressed on T cells. EC$_{50}$ values for MSLN-dependent target cell killing in the range of 0.6 to 15 pM suggest that formation of a cytolytic synapse between target and T cells required only minute concentrations of HPN536. Nevertheless, target cell lysis and T cell activation was of highest specificity as it was not observed with cancer cells lacking MSLN expression or with a TriTAC specific for GFP. HPN536 was similarly active across a variety of MSLN expressing human cancer cell lines from different cancer indications in vitro and in xenograft models and against cell lines engineered to express human or cynomolgus MSLN. Of note, HPN536 was designed to bind to mature MSLN as it is retained on the cell surface after release of MPF. This way, serum levels of MPF will not impact its activity. The high biological activity, stability and favorable PK properties of HPN536 support studies testing its clinical activity in patients with MSLN-expressing cancers.

Molecules bridging CD3ε on T cells with a target antigen on cancer cells can organize formation of synaptic structure at the cells’ interface that resembles that of a native immunological synapse as formed between a TCR complex on T cells with peptide-MHC complexes on antigen-presenting cells. Such cytolytic synapses have been described for a EpCAM/CD3-bispecific BiTE molecule (53). They serve as sites for T cell signal transduction and activation, and as sites for perforin and granzyme release ultimately leading to target cell lysis and caspase 3- and 7-mediated apoptosis(54). The EC$_{50}$ values for redirected lysis by HPN536 are several logs lower than the K$_D$ values measured for HPN536 binding to MSLN and CD3ε,
suggesting that only very few TriTAC molecules are needed to connect TCRs on T cells with MSLN on tumor cells for cytolytic synapse formation. Cooperative adhesion based on avidity gain and a limited diffusion of HPN536 out of the synaptic structure may be crucial for forming and stabilizing synapses with very few TriTAC molecules.

A close species cross-reactivity of HPN536 between human and cynomolgus monkey MSLN, albumin, and CD3ε provided the basis for a meaningful assessment of PK, pharmacodynamic (PD), and toxicology of HPN536 in the non-human primate species. While all tested doses led to a transient eclipse of lymphocytes, only the 10 mg/kg dose of HPN536 led to a more robust and transient cytokine release. While transient lymphocyte margination does not need T cell activation but may be largely governed by a conformational change of cell adhesion molecule LFA-1 on T cells to a high-affinity variant --referred to as in-side-out signaling (55) -- cytokine release and CD69 expression in monkeys was likely to have resulted from synapse formation between T cells and MSLN-expressing normal cells leading to new gene expression. The pharmacodynamic findings with HPN536 in non-human primates resemble those of other T cell engaging molecules (56,57). A possible target on normal tissue for HPN536 is the mesothelial cell layer lining cavities, the major tissue expressing MSLN. Reversible hyperplasia and immune infiltrates and matrix deposition observed in the mesothelial linings of treated monkeys support this notion. These dose-dependent histopathological findings suggest that HPN536 can reach the mesothelial layers and recruit T cells. Simultaneous engagement of MSLN and CD3ε on the mesothelial cells and T cells, respectively, by HPN536 then resulted in redirected lysis of the MSLN-expressing cell layer. As sequelae, other immune cells get attracted, and the mesothelial layer thickens possibly due to an ensuing fibrosis. Despite
changes at the microscopic level, HPN536 was remarkably well tolerated at single doses up to 10 mg/kg without gross macroscopic findings or dose-limiting toxicities. Of note, the $C_{\text{max}}$ at the 10 mg/kg dose exceeded the highest in vitro EC$_{50}$ value for redirected lysis (i.e., 15 pM observed in the HPAFII cells) by a factor of 400,000-fold. The PD and toxicology evaluation of HPN536 in the relevant non-human primate species suggest the potential for a wide therapeutic window.

In conclusion, the present pre-clinical characterization of HPN536 has provided (i) a scientific rationale to examine the activity of HPN536 in patients suffering from MSLN-expressing cancer, (ii) a further understanding of its potency and mechanism of action, (iii) a basis for calculating the first-in-human clinical dose for a Phase 1/2a clinical study based on the recommendation by Saber et al. (58), and (iv) a high safety margin in the absence of malignant tissue as shown in a pharmacological relevant non-human primate species. An open-label, Phase 1/2a study of HPN536 as monotherapy to assess the safety, tolerability and PK in patients with advanced cancers associated with mesothelin expression is currently ongoing (NTC 03872206).

**FIGURE LEGENDS**

**Figure 1. Binding of HPN536 to MSLN, albumin and CD3ε**

A, Schematic illustration of HPN536 as a trispecific molecule consisting of an anti-MSLN single domain antibody that targets cells expressing MSLN ($\alpha$MSLN), an anti-albumin single domain antibody that extends half-life ($\alpha$ALB), and an anti-CD3ε scFv that engages T cells ($\alpha$CD3ε). B, HPN536 affinity for three targets assessed by biolayer interferometry. Representative curves are shown for human MSLN (huMSLN), CD3ε (huCD3ε), and HSA (huALB). The red curves in
each panel represent a global fit of the data to a one to one binding model with decreasing concentrations indicated from top to bottom. The table summarizes HPN536 affinity to human, cynomolgus and mouse targets for replicate experiments. (NB, no binding). C, FACS binding of HPN536 to MSLN-expressing OVCAR8 and human T cells.

**Figure 2. HPN536 directs T cell killing in the presence of MSLN expressing cells**

A, Luciferase-labeled OVCAR3 cells were incubated with resting human T cells from five different donors at a 1:10 target-to-T cell ratio. Titrations of HPN536 or a negative control, anti-GFP TriTAC protein, were added to the target cell / T cell co-cultures. Forty-eight hours later, viability of the OVCAR3 cells was assessed by measuring luciferase activity. B, Luciferase-labeled, MSLN-expressing Caov3, Coav4, OVCAR3, and OVCAR8 and MSLN-negative MDAPCa2b and NCI-510a cells were incubated with resting human T cells at a 1:10 target-to-T cell ratio. Titrations of HPN536 were added to the target cell/T cell co-cultures. Forty-eight hours later, viability was assessed by measuring luciferase activity. C, Pancreatic cell lines HPAFII, Hs766T, CaPan2 and (D) NSCLC cell lines NCI-H596, NCI-H292, NCI-H1563 were incubated with resting human T cells at a 1:10 target-to-T cell ratio. Titrations of HPN536 or a negative control, an anti-GFP TriTAC protein, were added to the target cell / T cell co-cultures. Forty-eight hours later, viability was assessed by measuring luciferase activity.

**Figure 3. HPN536 directs T activation in the presence of MSLN expressing cells**

OVCAR8 ovarian cancer cells were incubated with resting human T cells from 4 different donors. Titrations of HPN536 or a negative control, anti-GFP TriTAC protein, were added to the
target cell/T cell co-cultures. Forty-eight hours later, expression of CD69 (A) and CD25 (B) were measured by flow cytometry. TNFα (C) or IFNγ (D) levels were also measured in the conditioned media collected from the co-cultures.

**Figure 4. HPN536 effectively inhibits the growth of multiple cancer types.**

**A,** HPAFII and T cells expanded from a healthy donor were co-implanted on day 0 at an E:T of 1:1 and treated with HPN536 (4, 20 or 100 ug/kg) or anti-GFP TriTAC (100 ug/kg) on days 1-15 (n=10). **B,** HPAFII and T cells expanded from a healthy donor were co-implanted on day 0 at an E:T of 1:1, allowed to grow for 7 days until they reached a volume of 170 mm$^3$. Mice were treated with HPN536 (100 or 500 ug/kg) and anti-GFP TriTAC (500 ug/kg) for 10 days (n=5). **C,** TOV21G cells were co-implanted with T cells expanded from a healthy donor at an E:T of 1:1 on day 0 and treated with HPN536 (20, 100 or 500 ug/kg) or anti-GFP TriTAC (500 ug/kg) on days 1-15 (n=10). **D,** NCI-H292 were co-implanted with PBMCs from a healthy donor at an E:T of 1:1, allowed to grow for 6 days at which point the tumor volume reached 27 mm$^3$. Treatment with HPN536 (20, 100 or 500 ug/kg) or vehicle was initiated on day 6 and continued daily to day 16 (n=8). Upward pointing arrows indicate dosing. One-way ANOVA followed by Tukey’s post hoc test using Prism Version 7 software was used for statistical analyses.

**Figure 5. A single dose of HPN536 was well tolerated with evidence of target engagement in cynomolgus monkeys**

**A,** HPN536 serum concentrations after a single intravenous bolus dose to cynomolgus monkeys at doses of 0.1, 1, or 10 mg/kg. For the functional assay, HPN536 was captured with
biotinylated CD3ε and subsequently detected with a sulfo-tagged mesothelin (MSLN). For the anti-idiotype assay, HPN536 was captured with an anti-idiotype antibody recognizing the anti-albumin domain and was detected with a sulfo-tagged CD3ε. B, TDCC comparing the activity of HPN536 in cynomolgus serum PK samples 168 hours post dose, stock HPN536 and control anti-GFP TriTAC. C, Transient, dose dependent, T lymphocyte reduction as demonstrated by flow cytometry analysis of total T cells in blood. D, Transient CD69 activation in T lymphocytes by 10 mg/kg HPN536 8 hours post dose. E, Serum IL-6 cytokine expression following HPN536 administration in cynomolgus monkeys. LLOQ (lower limit of quantitation).

Figure 6. MSLN expressing tissues display reversible hyperplasia and inflammation after HPN536 dosing

The left panel shows vehicle control treated lung sample. Middle panel shows lung sample at necropsy 8 days post HPN536 treatment. Right panel shows lung sample at necropsy 22 days post HPN536 treatment. MSLN expressing tissues display reversible hyperplasia and inflammation after HPN536 dosing. Yellow arrow points to mesothelial border. Red arrow points to immune infiltrates. Green arrow points to extracellular matrix deposition (fibrin and/or collagen).

Table 1. Summary of EC_{50} values from TDCC assays and MSLN sites per cell in different cell lines

Luciferase labeled target cells were incubated with resting human T cells at a 1:10 target-to-T cell ratio. Titrations of HPN536 or a negative control, a GFP-targeting TriTAC protein, were
added to the target cell/T cell co-cultures. Forty-eight hours later, viability of target cells was assessed by measuring luciferase activity. EC$_{50}$ values are expressed in pM concentrations. The QIFIKIT (Dako) was used according to manufacturer’s instructions to estimate the number of MSLN molecules expressed per cell.
REFERENCES


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<th>Tumor origin</th>
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**Figure 1.** Binding of HPN536 to MSLN, albumin and CD3ε.

### In vitro affinity measurements

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**A**
- αMSLN
- αALB
- αCD3ε

**B**

**C**
- HPN536 Binding to MSLN-expressing OVCAR8 cells
- HPN536 Binding to Human T cells

**Legend**
- HPN536
- Anti-GFP TriTAC
- Secondary control

Alexa fluor 647
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Mary Ellen Molloy, Richard J Austin, Bryan D Lemon, et al.

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