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Research Article

Preclinical evaluation of IAP0971, a novel immunocytokine that binds specifically to PD1 and fuses IL15/IL15Rα complex

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ABSTRACT

Background: Currently, cytokine therapy for cancer has demonstrated efficacy in certain diseases but is generally accompanied by severe toxicity. The field of antibody-cytokine fusion proteins (immunocytokines) arose to target these effector molecules to the tumor microenvironment to expand the therapeutic window of cytokine therapy. Therefore, we have developed a novel immunocytokine that binds specifically to programmed death 1 (PD1) and fuses IL15/IL15Rα complex (referred to as IAP0971) for cancer immunotherapy.
Methods: We report here the making of IAP0971, a novel immunocytokine that binds specifically to PD1 and fuses IL15/IL15Rα complex, and preclinical characterization including pharmacology, pharmacodynamics, pharmacokinetics and toxicology, and discuss its potential as a novel agent for treating patients with advanced malignant tumors.
Results: IAP0971 bound to human IL2/15Rβ proteins specifically and blocked PD1/PDL1 signaling transduction pathway. IAP0971 promoted the proliferation of CD8 + T cells and natural killer T (NKT) cells, and further activated NK cells to kill tumor cells validated by *in vitro* assays. In an hPD1 knock-in mouse model, IAP0971 showed potent anti-tumor activity. Preclinical studies in non-human primates following single or repeated dosing of IAP0971 showed favorable pharmacokinetics and well-tolerated toxicology profile.
Conclusion: IAP0971 has demonstrated a favorable safety profile and potent anti-tumor activities *in vivo*. A Phase I/IIa clinical trial to evaluate the safety, tolerability and preliminary efficacy of IAP0971 in patients with advanced malignant tumors is on-going (NCT05396391).

Statement of Significance: IAP0971 is a novel bifunctional PD1/IL15 immunocytokine that has demonstrated excellent anti-tumor efficacy and favorable safety profile, which has the potential to address both primary non-responsiveness and acquired resistance of current immunotherapy.

KEYWORDS: PD1; IAP0971; IL15; immunocytokine; cancer immunotherapy

INTRODUCTION

Cytokines are natural substances made by cells, which have been used in cancer treatment for years. Interferon-alpha (IFNα) and interleukin-2 (IL2) were the first cytokines approved for cancer therapy [1–3]. In addition, there were other cytokines used in cancer immunotherapy, such as IL6, IL15, IL10 and so on [3]. However, cytokine immunotherapy has encountered severe dose-limiting side effects [4], which negatively affected its clinical applications. Capillary leak syndrome and cytokine release syndrome

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were well-known [5–7]. In addition, the short half-life of cytokines was another disadvantage [8].

Interleukin-15 (IL15) is a glycoprotein with a molecular weight of 14–15 kDa, consisting of 169 amino acids [9], which is a very potent immune cell activator. IL15 and IL2 share two common receptors, IL2/15Rβ and IL2/15Rγ which are mainly expressed on T cells and NK cells. Currently, single-chain IL15 and several heterodimeric IL15:IL15Rα variants (hetIL15, N-803 and RLI) were being tested in clinical trials [10].

Programmed death 1 (PD1) and its ligands, PD-L1 and PD-L2, transmit inhibitory signals that coordinate the balance between T cell activation, tolerance and immunopathology [11]. U.S. Food and Drug Administration (FDA) approved two PD1 blockade antibodies: pembrolizumab (Keytruda) and nivolumab (Opdivo) in 2014 [12]. In recent years, PD1/PDL1 immunotherapy has become one of the most popular research and development directions in the world, and has been approved for over 30 indications [13]. According to the U.S. clinical trial database, there has been over 1 000 clinical trials of PD1-related therapy [14]. Despite these breakthroughs, the majority of patients failed to respond to current immune checkpoint blockade and demonstrated primary resistance. In addition, many of those who initially responded to the treatment eventually relapsed to develop acquired resistance [15, 16].

IAP0971 is a novel heterodimeric fusion protein that specifically binds to PD1 and fuses the cytokine IL15/IL15Rα complex, which belongs to a kind of immunocytokines. The anti-PD1 antibody in IAP0971 released the immune inhibition of PD1/PDL1 axis, which meanwhile increased the targeting of IL15 to the tumor microenvironment and prevented the systematic non-specific activation. IL15 in IAP0971 can stimulate, activate and essentially enhance the infiltration of CD8 + T cells and NK cells in the tumor microenvironment to further exert anti-tumor activities. Besides, IAP0971 has an antibody-based structure and a longer half-life than that of the recombinant IL15 or IL15-Fc fusion protein, which can delay the metabolism of the drug in the blood and make the administration cycle longer. In summary, IAP0971 is a novel bifunctional PD1/IL15 immunocytokine that has demonstrated excellent anti-tumor efficacy and favorable safety profile, which has the potential to address both primary non-responsiveness and acquired resistance of current immunotherapy.

MATERIALS AND METHODS

Binding of IAP0971 to PD1 and IL2/15Rβ

Test article IAP0971 was generated at SunHo (China) BioPharmaceutical Co., Ltd. PD1 and IL2/15Rβ proteins (ACRO Biosystems) were diluted and coated into 96-well plate for overnight at 4°C. The plates were blocked with blocking solution for 1 h. IAP0971 and isotype control were diluted and applied to the enzyme-linked immunosorbent assay (ELISA) plate at 37°C for 1 h. After washing the plate, secondary antibody was added for 45 min. Followed by washing, the plate was then developed with Tetramethylbenzidine (TMB) peroxidase substrate and terminated with 1 M HCL. Absorbance at 450 nm (A450) was determined.

PD1/PDL1 blockade bioassay

CHOK1-PDL1 TCR cells (HankeMab Therapeutics) were seeded at 5×10^4 cells per well in 96-well white plates (WHB; WHB-96-01) and incubated overnight in a 5% CO_2 incubator at 37°C. The next day, Jurkat-NFAT-Luci-PD1 cells (HankeMab Therapeutics) were seeded at 5×10^4 cells per well with pre-plated cells or without cells. Serially diluted IAP0971 was incubated with co-cultured cells or only Jurkat-NFAT-Luci-PD1 cells for 6 h in a 5% CO_2 incubator at 37°C. After that Bio-Lite Luciferase Assay System (Vazyme, DD1201–02) was added, after which chemiluminescence was detected on a microplate reader (TECAN) and analyzed using GraphPad.

PBMC proliferation assay

Peripheral blood mononuclear cells (PBMC) (Oricells) were seeded in 24-well plates (1 × 10E6 cells/well, for cell proliferation assay) in the presence of 1 μg/ml OKT3 (BioGems, 10311–25-500) and cultured for 24 h in a 5% CO_2 incubator at 37°C. After that, serially diluted IAP0971, IL-15Rα&IL-15 complex (Novoprotein Scientific Inc., CK94), Keytruda (trade name for the generic drug pembrolizumab, Merck) and human IgG4 isotype control (Sino Biological Inc., HG4K) were incubated with PBMC. Cell number was counted by a coulter counter (Thermo Fisher Scientific;16–9-002) on Days 3, 6, 9, 12. On Day 12, proliferative cells were stained with percp-cy™5.5 mouse anti-human CD3 (BD, 552852), APC/cy7 anti-human CD4 (Biolgend, 357416), FITC anti-human CD8 (Biolgend, 980908) and anti-CD56 (BD, 555518) for flow cytometry (FACS) analysis.

NK cell cytotoxicity assay

NK cells were isolated from PBMC (Oricells) using the EasySep™ Human NK Cell Isolation kit (STEMCELL, 17915) according to the manufacturer's protocol. NK cells were cultured with 1 nM IAP0971 or human IgG4 isotype Control in 24-well plates (Thermo Fisher Scientific, 142475) overnight in a 5% CO_2 incubator at 37°C. OVCAR3 cells (National Collection of Authenticated Cell Cultures) were seeded at 1×10^4 cells/well into 96-well U-bottom plate (Thermo Fisher Scientific, 163320). Pre-activated NK cells were added at the following effector-to-target (E:T) ratios: 5:1, 2:1. After 4 h of incubation at 37°C, the specific lysis was evaluated using an lactate dehydrogenase (LDH) release assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega) according to the manufacturer's protocol. LDH release in the supernatants was measured by a microplate reader (TECAN). The percentage of specific lysis was calculated: 100 × (experimental release—spontaneous release)/(maximum release—spontaneous release). In the denominator, the maximum release and spontaneous release refer to that of the target cells (OVCAR3 cells in this case); in the numerator, the experimental release and spontaneous release refer to that

of the combined effector and target cells (NK cells and OVCAR3 cells in this case) with or without test articles, respectively. Since there was no significant death of NK cells in this assay, the assay measured the specific lysis of target cells.

Animal husbandry

C57BL/6-hPD-1 mice were kept in individual ventilation cages at constant temperature and humidity (20–26°C, 40–70% relative humidity) (WuXi AppTec (Shanghai) Co., Ltd). Cynomolgus monkeys (*Macaca fascicularis*) weighing ~2.3–3.6 kg were used for PK and toxicity studies, housed in stainless steel cages in a controlled environment (18–26°C, 40–70% relative humidity) on a 12-h light/dark cycle at various facilities where the in-life portion of the studies was conducted (WuXi AppTec (Suzhou) Co., Ltd). The care or use of animals had been reviewed and approved by Institutional Animal Care and Use Committee (IACUC).

In vivo anti-tumor activity in tumor xenograft model

Cell line-derived xenograft model was established by subcutaneous (SC) inoculation of $\sim 3 \times 10^5$ MC38-hPD-L1 cells into C57BL/6-hPD-1 mice (WuXi AppTec (Shanghai) Co., Ltd). At 7 days post implantation, mice were randomized with mean tumor volumes calculated at ~65 mm^3 (10 animals per group), and then dosed intraperitoneally with 0.1, 0.5 or 1 mg/kg IAP0971, phosphate-buffered saline (PBS) or 0.5 mg/kg Keytruda, three times a week for 8 weeks. Tumor size was measured twice a week throughout the study.

Single-dose pharmacokinetic (PK) study

In the single-dose study, 18 male/female monkeys (WuXi AppTec (Suzhou) Co., Ltd) were assigned to three groups. Animals were administered with a single dose of 0.2 mg/kg IAP0971 by intravenous (IV) bolus administration or 0.2, 1 mg/kg IAP0971 by SC administration on Day 1. Blood samples for PK analysis were collected from each animal predose and at 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 168, 240, 336 and 554 h postdose.

Repeated-dose: 5-week toxicity and pharmacodynamic study

For the 5-week repeated-dose toxicity study, a total of 40 cynomolgus monkeys (20/sex) were randomly assigned into 4 groups of 5/sex/group and received IAP0971 once weekly at doses of 0 (control), 0.2, 0.6 or 1.2 mg/kg by SC injection for 5 consecutive weeks. All available animals (3 monkeys/-sex/group) from dosing phase were terminated on study Day 30. All recovery animals (2 monkeys/sex/group) were necropsied at the completion of a 5-week recovery period after cessation of dosing (Day 65) to assess the reversibility of any potential treatment-related effects.

Criteria for evaluation included viability (mortality and moribundity), clinical observations (including local tolerance at injection sites), body weights, qualitative food consumption, ophthalmology, body temperature, safety pharmacology (electrocardiography, blood pressure and heart rate, respiratory and neurological examinations), clinical pathology (hematology, serum chemistry, coagulation and urinalysis), toxicokinetics (TK), immunogenicity (anti-drug antibody) analysis, immunophenotyping, cytokine (IL2, IL6, IFNγ and TNFα) analysis, receptor occupancy analysis, immunoglobulin and complement analysis, gross necropsy, organ weights and histopathology.

PK assay methods

An indirect antigen ELISA assay was used for the detection of IAP0971 in cynomolgus monkey serum (WuXi AppTec (Suzhou) Co., Ltd). The capture agent was human PD1/PDCD1 Protein, His Tag (ACRO, PD1-H5221), coated onto 96-well ELISA plates. After overnight incubation, the plates were blocked followed by the addition of the samples. Any IAP0971 present in the sample will be bound by the coating reagent. After washing away any unbound substances, mouse anti-Human IgG Fc-HRP (Southern Biotech, 9040–05) was added into the well to bind with IAP0971. Following a wash to remove any unbound HRP, a substrate was added to the wells and then reacted with the peroxidase to create a colorimetric signal that was proportional to the amount of IAP0971.

Receptor occupancy (RO) assay

Receptor occupancy assays were conducted by flow cytometry on Cytotoxic T cells and NK cells. Blood samples from the above repeated-dose toxicity study were collected at predose, 4, 24 and 72 h postdose on Day 1 and Day 22. Each blood sample was divided into two tubes and preincubated with a saturating concentration of unlabeled Fc of IAP0971 (control article) or IAP0971 (test article), respectively, and then co-stained with PE-streptavidin (BD, 554061), Anti-human IgG4 pF_C (HP6023) (Biotin) (Abcam, ab99818), FITC-conjugated anti-NHP CD45 (BD, 557803), V450-mouse anti-human CD3 (BD, 560351), PerCP-Cy™5.5 Mouse Anti-Human CD4 (BD, 552838), Brilliant Violet 510™ anti-human CD16 (Biolegend, 302048) and APC-Cy7-conjugated anti-human CD8 antibodies (BD, 561423) for 30 min at 2–8°C in the dark. The erythrocytes were lysed in 2 mL 1× BD FACS Lysing Solution (BD, 349202) for 10–12 min. The receptor occupancy rate (%RO) was calculated as follows: %RO = (MFI of control article)/(MFI of test article) × 100%. The control article unlabeled Fc of IAP0971 was prepared by removing Fab part of IAP0971 through enzyme digestion. The receptor occupancy signal of effector cells collected in cynomolgus monkeys was calculated by comparing the experimental signal with saturation signal. The saturation signal was measured by saturating the effector cells with excessive IAP0971. The experimental signal should be measured by adding non-specific IgG4 isotype control, or was measured by adding unlabeled Fc of IAP0971 in this case. Due to the difference in non-specific binding of test article and control article, the receptor occupancy presented may have some uncertainty, especially for lower number of NK cells.

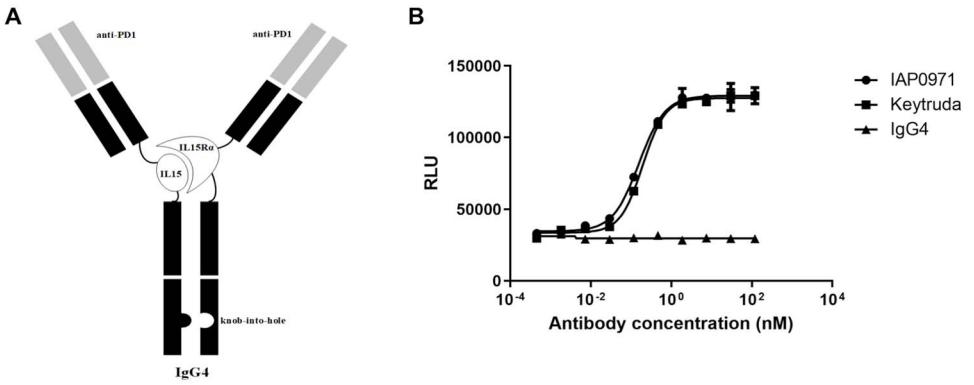

Figure 1. Basic characteristics of IAP0971. (A) Schematic structural view of IAP0971 molecule. (B) Reporter gene PD1/PDL1 blockade bioassay was performed to estimate the ability of IAP0971 to block PD1/PDL1 signaling and T cell activation. All samples were duplicated or triplicated.

Tissue cross-reactivity

An immunohistochemistry method was employed to evaluate the tissue cross-reactivity of biotinylated test article (Biotin-IAP0971), and biotinylated control article (Biotin-Human IgG4) in cryo sections from a full panel of human and cynomolgus monkey tissues (each kind of tissue from 3 individuals). Frozen normal human tissues were commercial materials from national disease research interchange/Asterand bioscience/BioIVT. Normal cynomolgus monkey tissues were collected from naïve animals of Hainan Jingang Biotech Co., Ltd, and frozen in tissue-tek optimal cutting temperature compound maintained at $<-60°C$ by the testing facility. PBS was used as reagent control. CHOK1-PD1 cells were used as the positive control and CHOK1 cells were used as the negative control. The testing facility validated the freshness and adequacy of the tissue samples by staining sections of all tissue samples with a monoclonal antibody directed against CD31 antigen by immunohistochemistry. All tissues were evaluated with two different concentrations (Biotin-IAP0971 or Biotin-Human IgG4, 5 mg/mL (the optimal binding concentration) and 15 mg/mL (3 × the optimal binding concentration).

RESULTS

Binding and blocking activity of IAP0971

IAP0971 is a heterodimeric fusion protein that binds specifically to human PD1 and fuses the natural human cytokine IL15/IL15Rα complex. IAP0971 consists of four chains, an anti-PD1 antibody heavy chain fused with cytokine IL15 defined as heavy chain 1 (HC1), an anti-PD1 antibody heavy chain fused with cytokine receptor IL15Rα defined as heavy chain 2 (HC2), the other two identical anti-PD1 antibody light chains defined as LC (Fig. 1A). IL15 and IL15Rα sushi domain were placed in the hinge region of HC1 and HC2, respectively. Classical disulfide bonds were kept, and knobs-into-holes mutations were introduced to the Fc region for heterodimer formation. IAP0971 formed a normal IgG structure with cytokine fusion, as validated by the denaturing and non-denaturing SDS-PAGE analysis (data not shown).

Binding affinity of IAP0971 to PD1 and IL2/15Rβ proteins of different species was analyzed by ELISA. The ELISA test results showed that IAP0971 could bind to human and monkey PD1 proteins, but not to mouse PD1 protein (Table 1). IAP0971 bound to human and monkey IL2/15Rβ proteins, as well as mouse IL2/15Rβ protein, even though with slightly lower affinity (Table 1). We have further validated that IAP0971 can simultaneously bind to PD1 and IL2/15Rβ in both ELISA and cell-based FACS assays (Supplementary Figs 1 and 2). IAP0971 can direct IL15 activity to PD1+ cells, especially CD8+ tumor-infiltrating T lymphocytes, instead of systematic activation by IL15 or IL2 molecules, as recently reported [17, 18].

Reporter gene assay was used to detect the blocking activity of IAP0971. The results showed that IAP0971 can efficiently block the binding of human PD1 on the surface of T cells to human PDL1 on the surface of CHO cell, and transmit the activation signal of T cells. The EC_{50} was 0.16 nM (Fig. 1B). The blocking activity of IAP0971 was similar with that of Keytruda. These results indicated that IAP0971 can release the immune inhibition of PD1/PDL1 axis and activate immune effector cells for anti-tumor activities.

PBMC proliferation and NK cytotoxic activity of IAP0971

After pre-activation of PBMC with OKT3, stimulation of IAP0971 on PBMC proliferation was detected on Days 3, 6, 9, 12 by the cell counting method. The results showed that IAP0971 and IL15Rα&IL15 could stimulate PBMC proliferation in a time and concentration dependent manner, which was not observed for Keytruda, indicating that the stimulation of IAP0971 on PBMC proliferation depended on the IL15/IL15Rα complex in the molecule (Fig. 2A–D). To be noted, the IL15 stimulation activity in IAP971 was decreased by about 5-fold compared with IL15/IL15Rα precomplex in solution due to the steric hindrance of IAP0971 structural design, to match the balanced dose of PD1 antibody and IL15/IL15Rα complex. On Day 12, flow cytometry data revealed IAP0971 and IL15Rα&IL15 precomplex mainly stimulated the proliferation of CD8 + T (CD3 + CD8 + CD4−) and NKT cells (CD3 + CD56 + CD8+) strongly, but not CD4 + T

Table 1. Binding activity of IAP0971 to PD1 and IL2/15Rβ proteins of human, mouse, monkey

Species	PD1 (nM)			IL2/15Rβ (nM)	
	Keytruda	IAP0971	IgG1 Control	IAP0971	IgG4 control
Human	0.06557	0.06338	No binding	12.29	No binding
Mouse	No binding	No binding	No binding	170.4	No binding
Monkey	0.07052	0.06927	No binding	3.666	No binding

cells (Supplementary Table 1). Some research has suggested that IL15 can enhance NK cell cytotoxicity, and therefore pre-activated NK cells by IAP0971 were co-incubated with OVCAR3 cells for 4 h at different effector-target cell ratios (Fig. 2E). The results showed that OVCAR3 cells were killed in presence of IAP0971 pre-activated NK cells. Moreover, the higher the effector-target cell ratio, the stronger the killing effect. Taken together, IAP0971 showed the capability to stimulate CD8 + T and NKT cells proliferation and activate NK cells to kill tumor cells.

Anti-tumor efficacy in hPD1 knock-in mice

In vivo anti-tumor efficacy of IAP0971 was evaluated in a human PD1 knock-in tumor mouse model of MC38-hPDL1 colon adenocarcinoma. Mean tumor volumes were calculated on Day 31 post-tumor cell implantation (Fig. 3A–F). Compared with the control group, IAP0971 had a significant tumor growth inhibition (TGI) effect at doses of 0.1, 0.5 and 1 mg/kg, with TGI of 106.13%, 110.47% and 103.56%, respectively. In the IAP0971 0.1 mg/kg group, eight animals achieved complete remission; nine animals in the 0.5 mg/kg group achieved complete remission; nine animals in the 1 mg/kg group achieved complete remission (Supplementary Fig. 3). There was no significant difference among the 0.1 mg/kg group, the 0.5 mg/kg group and the 1.0 mg/kg group, which all showed great TGI, probably due to the extremely high potency of IAP0971. At the same dose of 0.5 mg/kg, the anti-tumor effect of IAP0971 was better than that of Keytruda, and the complete tumor remission rate increased by 40%. Notably, there was no obvious body weight change across all IAP0971 dosing groups compared with control groups, demonstrating the safety of IAP0971 (Fig. 3G). Taken together, IAP0971 showed strong anti-tumor activities with excellent safety as a novel immunocytokine.

Single-dose pharmacokinetic study

PK analysis of IAP0971 was undertaken in cynomolgus monkeys following single IV infusion at 0.2 mg/kg and SC injection at 0.2, 1.0 mg/kg dosages (Table 2). For the IV infusion administration of IAP0971 at 0.2 mg/kg, IAP0971 showed a serum clearance (Cl) of 0.0513 ± 0.00732 mL/min/kg, half-life ($T_{1/2}$) at 10.5 ± 1.86 h. The volume of distribution (Vd_{ss}) was 0.0465 ± 0.00541 L/kg, the area under the serum concentration-time curve ($AUC_{0\text{-last}}$) value was 61 100 ± 10 300 ng·h/mL. The max concentration (C_{max}) was 4 180 ± 367 ng/mL, the time to reach C_{max} (T_{max}) was 2.00 h. Following SC administration of IAP0971 at 0.2 and 1 mg/kg, $AUC_{0\text{-last}}$ values of IAP0971 were 27 900 ± 5 610 and 231 000 ± 284 00 ng·h/mL, respectively. The C_{max} values were 847 ± 173 and 5 280 ± 410 ng/mL, whereas T_{max} reached at 14.0 ± 7.90 and 12.7 ± 5.89 h, respectively. The SC bioavailability was 45.7 and 75.6% at 0.2 and 1.0 mg/kg, respectively. The half-life ($T_{1/2}$) was 15.7 ± 6.67 h at 1.0 mg/kg(SC). The systemic exposure ($AUC_{0\text{-last}}$ and C_{max}) of IAP0971 increased with the increase of the dose among 0.2–1 mg/kg. As shown, there were no obvious sex differences at any dose levels when comparing the $AUC_{0\text{-last}}$ and C_{max} in female and male cynomolgus monkeys (Supplementary Table 2). These data demonstrated that SC administration has a moderate drug absorption while keeping a decent bioavailability and is preferred in clinical administration.

IAP0971 has a half-life around 15-fold longer than that of recombinant IL15, and about 2-fold longer than that of Anktiva [19, 20]. Compared with Keytruda, IAP0971 has a shorter half-life possibly due to ADA incidence and faster IL2/IL15R$\beta\gamma$-mediated clearance [17, 21]. Since IAP0971 is a potent immune system stimulator, the relative short half-life compared with traditional antibodies, and the relative long half-life as well as tumor-microenvironment targeting of IAP0971 compared with traditional cytokines may bring additional benefits of safety profile for larger therapeutic window.

Repeated-dose toxicity and pharmacodynamic study

Cynomolgus monkeys received IAP0971 at doses of 0.2, 0.6 or 1.2 mg/kg/dose once weekly for a total of five doses following a 5-week recovery period. TK showed that the Tmax values for IAP0971 were observed between 4.0 and 48.0 h postdose, $T_{1/2}$ values ranged from 8.5 to 19.6 h. No marked sex difference in systemic exposure was observed at any dose level. The systemic exposure increased dose-proportionally in both sexes on Day 1, but increased more than dose-proportionally in females on Day 22 which may be affected by ADA (Table 3). Similar ADA incidence of IAP0971 was observed as that of Keytruda and IL15/IL15Rα-Fc superagonist (N-803) previously reported in macaques (data not shown). Considering the difference between macaques and humans, we would expect a lower ADA incidence rate of IAP0971 in human trials compared with that observed in preclinical macaque studies, which is the case for both Keytruda and N-803.

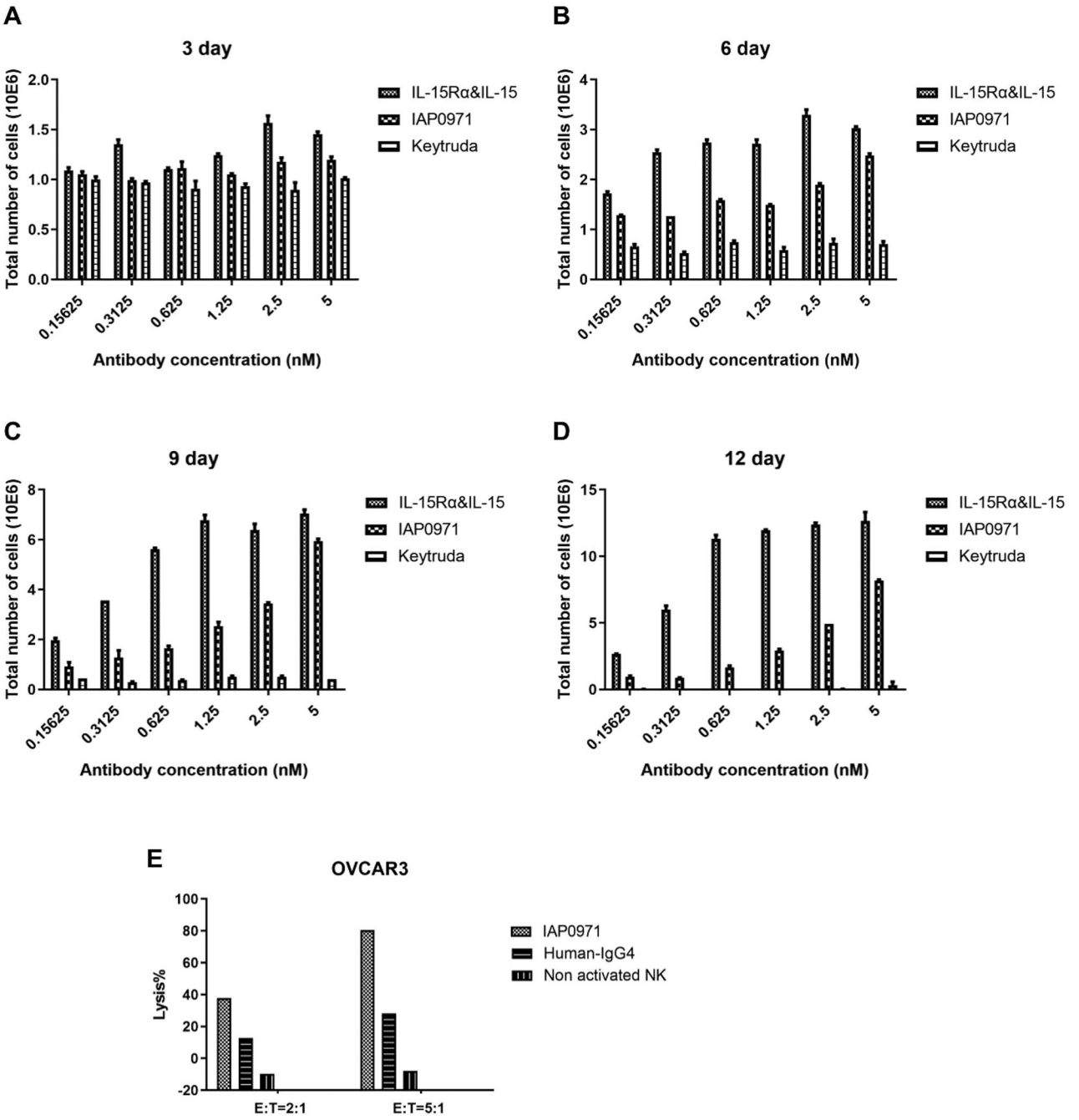

Figure 2. IAP0971 stimulated PBMC proliferation and activated NK cell cytotoxicity against OVCAR3 cells. The total number of cells was shown after pre-activation of PBMC with OKT3 (1 μg/mL), and stimulated with serially diluted from 5 to 0.15625 nM of IL15Rα&IL15, IAP0971 or Keytruda respectively, for (A) 3 days, (B) 6 days, (C) 9 days and (D) 12 days. (E) NK cell cytotoxicity assay was performed using OVCAR3 cells as target cells. IAP0971 or human IgG4 isotype control was added at a concentration of 1 nM for NK cell activation. NK cell cytotoxicity was measured at the following E:T ratios: 5:1 and 2:1. All samples were duplicated.

For hematology, during the dosing phase, when compared with predose or control group, dose-related increased leukocyte, lymphocyte and basophil were noted in both sexes at 0.2, 0.6 and 1.2 mg/kg/dose before the second, third and fifth dosing, and decreased at 24 h postdose. Decreased erythrocyte count, hemoglobin and mean corpuscular hemoglobin were noted in females at 0.6 and 1.2 mg/kg/dose before the second, third and fifth dosing. These changes also occurred in males, but the magnitude of change was less than that in females. For serum factors, at the end of dosing phase, decreased albumin in both sexes and increased total bilirubin in males were noted at 0.6 and 1.2 mg/kg/dose, which correlated with infiltration of mononuclear cells accompanied with mild hepatocellular necrosis, and/or minimal or mild hemorrhage in the liver. Complement C3c was increased after the fourth dosing

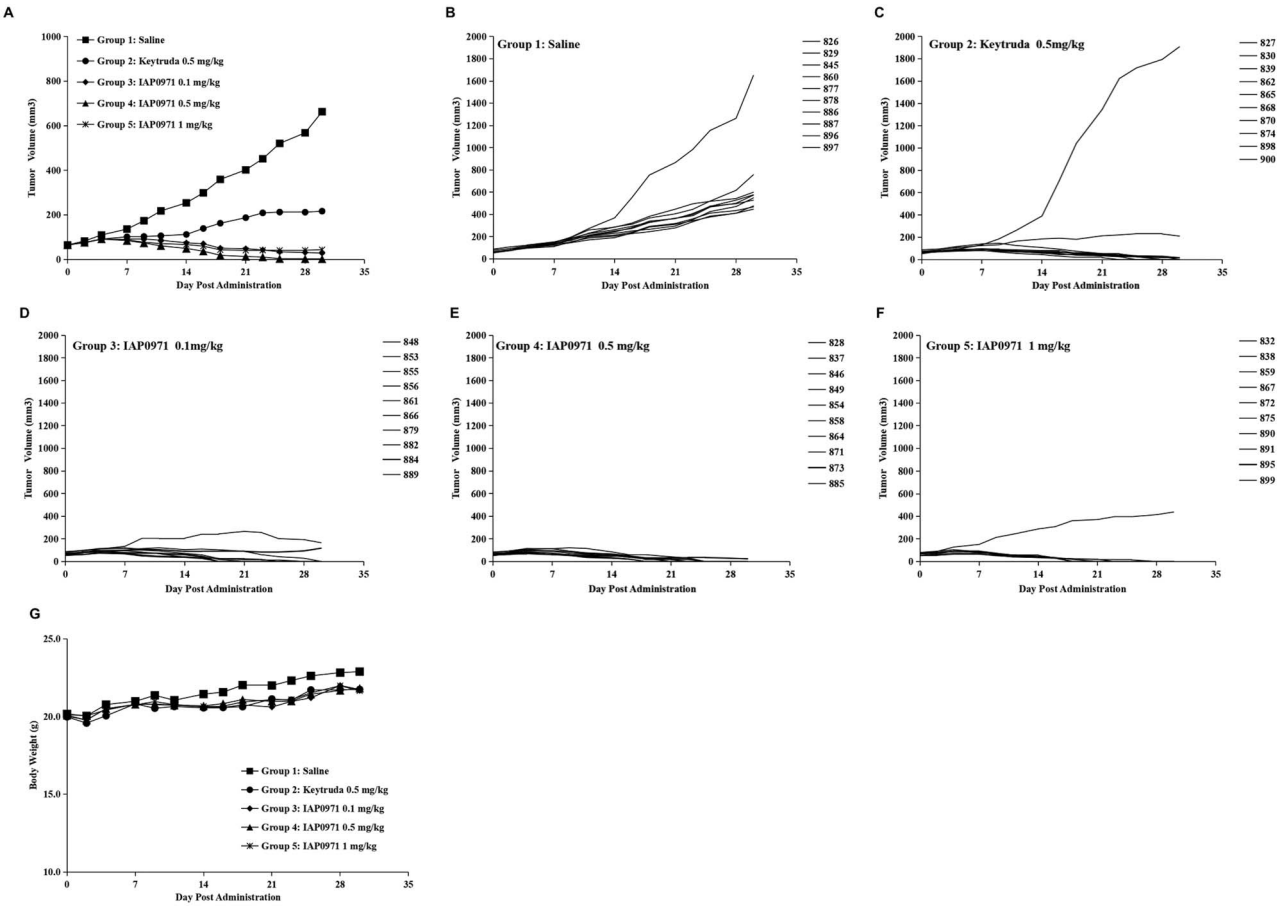

Figure 3. *In vivo* anti-tumor activity of IAP0971. (A) Average tumor volume. Human PD1 knock-in mice bearing MC38-hPDL1 tumors cells were treated with (B) Group 1: saline, (C) Group2: Keytruda 0.5 mg/kg or (D–F) IAP0971 0.1, 0.5, 1 mg/kg on a Q3D × 8 regime, and the tumor volume of each mouse was demonstrated with 10 mice in each group. (G) Average body weight was shown for each group.

Table 2. Pharmacokinetic parameters of single IV infusion/SC injection of IAP0971 in cynomolgus monkeys

Parameter	0.2 mg/kg (IV)	0.2 mg/kg (SC)	1 mg/kg (SC)
C_{max} (ng/mL)	4 180 ± 367	847 ± 173	5 280 ± 410
T_{max} (h)	2.00	14.0 ± 7.90	12.7 ± 5.89
$T_{1/2}$ (h)	10.5 ± 1.86	ND	15.7 ± 6.67
Vdss (L/kg)	0.0465 ± 0.00541	—	—
CL (mL/min/kg)	0.0513 ± 0.00732	—	—
$AUC_{0\text{-last}}$ (h·ng/mL)	61 100 ± 10 300	27 900 ± 5 610	231 000 ± 28 400
Bioavailability (%)[a]	—	45.7	75.6

[a]Bioavailability was calculated using AUC0-last and nominal dose. "ND" means not determined.
"—" means not calculated.

in both sexes at 0.6 and 1.2 mg/kg/dose. Absolute count of CD8 + T cells and NK cells decreased 24 h post each dose (except for post 4th dose which was untested) when compared with predose which was also observed in previous literature of IL15 studies [22], and increased significantly prior to the next dose (Supplementary Figs 4 and 5). In addition, test article-related increase of IL6 was noted but without dose-dependency, which were reversible (Supplementary Table 3). No significant changes were observed for other cytokines, including TNFα, IFNγ and IL2 (data not shown). The above changes did not show abnormality during the recovery phase. No toxicologically significant changes in cardiovascular, respiratory, or neurologic assessment were noted.

Table 3. TK parameters of repeated SC injection of IAP0971 in cynomolgus monkeys

Dose (mg/kg/dose)	Study Day	Sex	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	$AUC_{0\text{-}168h}$ (h∗ng/mL)
0.2	1	Male	786 ± 197	12.0 (4.0–12.0)	16.8	25 900 ± 8 310
		Female	949 ± 272	12.0 (12.0–24.0)	15.8 (12.9–19.6)	38 800 ± 9 800
	22	Female[a]	193	36.0	ND	7 800
0.6	1	Male	2 640 ± 213	12.0 (12.0–24.0)	15.5 (12.8–17.6)	112 000 ± 17 900
		Female	2 620 ± 855	12.0 (12.0–24.0)	14.5 (13.6–19.5)	110 000 ± 16 600
	22	Male	1 420 ± 530	12.0 (12.0–12.0)	ND	34 800 ± 13 000
		Female	902 ± 883	18.0 (12.0–24.0)	ND	28 700 ± 21 600
1.2	1	Male	6 620 ± 1 670	12.0 (12.0–24.0)	12.1 (8.5–14.6)	288 000 ± 46 500
		Female	4 980 ± 1 050	24.0 (12.0–24.0)	11.5 (8.8–18.0)	230 000 ± 34 100
	22	Male	4 030 ± 1 700	12.0 (12.0–24.0)	ND	99 900 ± 34 500
		Female	3 870 ± 797	12.0 (12.0–24.0)	ND	109 000 ± 17 500

ND: Not determined.
[a]Mean values for C_{max}, T_{max} and individual values for $AUC_{0\text{-}168h}$ On Day 22, the concentrations of all male animals were BLQ at 0.2 mg/kg/dose which may be affected by ADA, no $AUC_{0\text{-}168h}$ were determined.

Taken together, all animals were well tolerated to IAP0971 repeated-dose administration, even at doses 80-fold higher than N-803 [23].

Receptor occupancy

On Day 1, compared with the control group, the receptor occupancy of cytotoxic T cells and NK cells in all groups increased and reached its maximum at 4 h, then decreased at 24 h slowly, continued to 72 h postdose. At 72 h after administration, the receptor occupancy rate of cytotoxic T cells and NK cells was 56.73%–67.63% and 52.73%–100.99%, respectively, which were at a high level (Fig. 4A and B).

On Day 22, compared with the control group, the receptor occupancy of cytotoxic T cells and NK cells in the low-dose group did not increase at each detection point, which may be related to the rapid clearance and the lower blood concentration of the drug in the low-dose group after multiple doses. The cytotoxic T cells and NK cells showed an increase of receptor occupation at 0.6 and 1.2 mg/kg and reached the highest position at 4–24 h postdose, and there was a positive dose-correlation (Fig. 4A and B). The current RO assay did not distinguish PD1 binding and IL15R binding, as we use anti-Fc secondary antibody to detect total IAP0971 binding. However, based on the relative affinity of IAP0971 to PD1 and IL15R (∼50-fold difference, Table 1), we would expect that the binding was mainly mediated by PD1 arm of IAP0971.

In summary, even though the half-life of IAP0971 was around 15 h, the receptor occupancy of effector CD8+ T cells and NK cells remained high at 72 h, and the proliferation of those effector cells continued for > 7 days, which may support a less frequent dosing in human clinical trials.

Tissue cross-reactivity

All tissues stained with the anti-CD31 monoclonal antibody showed positive staining, which indicated the adequacy and validity of the tissues. No staining was observed in the slides stained with the reagent control. Biotin-IAP0971 showed positive membranous staining in the Positive Control, CHOK1-PD1 cells and the staining intensity and frequency was "3+" and "frequent" at 5 μg/mL and 15 μg/mL. No Biotin-Human IgG4 staining was observed. No staining of Biotin-IAP0971 or Biotin-Human IgG4 was observed in the negative control, CHOK1 cells. All the above results indicated that the assay was specific and reproducible. At the concentrations of 5 and 15 μg/mL, Biotin-IAP0971 showed positive membranous staining in human and cynomolgus monkey tissues: In human tissues, staining on the membrane of lymphocytes from 1/3 tonsils (observed at 15 μg/mL only) and 1/3 lymph nodes was observed, and the staining intensity and frequency were "1+" and "rare". In cynomolgus monkey tissues, staining on the membrane of lymphocytes from 2/3 lymph nodes, spleens and tonsils, and 3/3 colons were observed, and the staining intensity and frequency were "1+" and "rare" to "occasional". There was no Biotin-IAP0971 staining in other human or cynomolgus monkey tissues. There was no Biotin-Human IgG4 staining observed in any human or cynomolgus monkey tissues tested.

Overall, positive membranous staining of Biotin-IAP0971 was observed on the lymphocytes from human and cynomolgus monkey tissues, which were consistent with the distribution of PD1 and IL2/15R in tissues and were considered on-target binding.

DISCUSSION

In this study, we described the *in vitro* activity, *in vivo* efficacy, pharmacokinetics and toxicity profiles of IAP0971, a novel bifunctional PD1/IL15 immunocytokine. IAP0971 showed good binding activities to PD1 and IL2/15Rβ proteins, as well as dose-dependent binding to CD8 + T and NK cells in PBMC. IAP0971 stimulated the proliferation of CD8 + T cells and NKT cells in PBMC in a concentration-dependent manner, and activated NK cells for efficient tumor cell killing. *In vivo* efficacy mouse

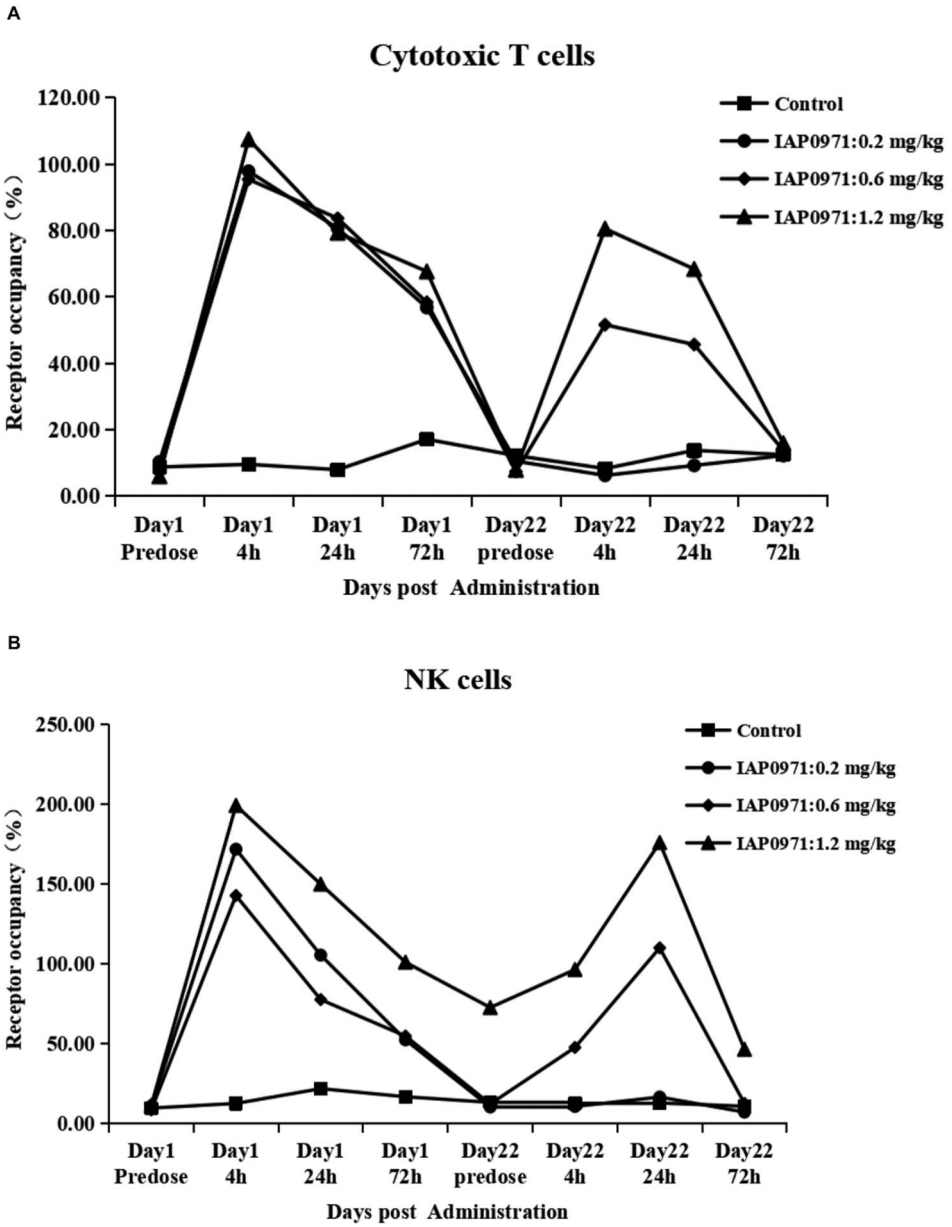

Figure 4. Receptor occupancy in repeat-dose toxicity study. Receptor occupancy at predose, 4 h, 24 h, 72 h on Day 1 and Day 22 in the cynomolgus monkey was calculated as follows: %RO = (MFI of control article)/(MFI of test article) × 100%. (A) Receptor occupancy on cytotoxic T cells. (B) Receptor occupancy on NK cells. The total IAP0971 binding was measured without distinguishing PD1 binding or IL15R binding.

model demonstrated that IAP0971 was extremely potent and showed better anti-tumor activities than benchmark Keytruda.

Following single IV or SC administration of IAP0971 into cynomolgus monkeys, no adverse effect was observed. IAP0971 showed no sex differences at all dose levels when comparing the $AUC_{0\text{-last}}$ and C_{max} in female and male cynomolgus monkeys. The $AUC_{0\text{-last}}$ and C_{max} of IAP0971 increased dose proportionally as dose levels increased from 0.2 to 1 mg/kg. In repeated-dose toxicity study, at the dose of 0.2–1.2 mg/kg, the counts of CD8 + T cells and NK cells transiently decreased 24 h after the administration, and the cell counts significantly increased in 7 days. The levels of IL6 increased slightly and other cytokine levels remained normal. The above drug-related changes were related to the proliferation of CD8 + T lymphocytes and NK cells caused by IAP0971, and were recoverable. All the animals were well tolerated at the end of the PK and toxicity studies.

Cancer immunotherapy has gained great breakthroughs since the 21st century, and brought significant clinical benefits to a large number of patients. However, even for the broadly used PD1/L1 immunotherapy, the relatively low objective response rate, short progression-free survival of patients, and drug resistance and recurrence especially for solid malignancy are the major clinical challenges [24–26]. The possible underlying biological explanation for those problems is that the microenvironment of different tumors has huge heterogeneity with significant differences in

immune cells infiltration and immune modulatory factors distribution. The exclusion, desertification and exhaustion of immune cells in the tumor microenvironment have been found and confirmed in many tumor cases, accounting for the major problems of current PD1/L1-oriented immunotherapy [27]. Cytokines, as naturally occurring immunomodulatory factors in the human immune system, play an essential role in transmitting signals and regulating the activity of immune cells to keep immune homeostasis. They are highly effective and respond very rapidly with a short half-life, exert significant biological effects even at low concentration. However, because of this, administration of cytokines exogenously has high systemic toxicity in terms of druggablity and very low therapeutic window even in combination therapy with other drugs [7]. This concern undoubtedly overshadows the development of cytokine-related drugs for cancer therapy. In recent years, there are single-chain recombinant IL15 and IL15/IL15R heterodimer (such as hetIL-15, N-803 and RLI) being investigated [10]. A safety study in rhesus monkeys of recombinant human IL15 (rhIL15) revealed half-life at 1.1 h and transient neutropenia at grade 3/4, with marrow cellularity increased, including observation of neutrophil in bone marrow and sinusoids of enlarged livers and spleens [19]. Compared with single-chain IL15, half-life of IL15/IL15R heterodimer was increased to about 4 h in a mice pharmacokinetic study [28]. The current optimization of cytokines for anti-tumor drug mainly includes PEGylation or Fc fusion for half-life extension, modulation of affinity by structural changes or amino acid mutations for receptor binding selectivity improvement, construction of cytokine and receptor complexes for efficacy enhancement and masking of cytokines as prodrug for onsite release [29, 30]. ALT-803, also called N-803, consist of IL15 mutant (IL15N72D) and IL15RαSu/Fc dimer protein complex, the half-lives were 7.5 h for IV and 7.7 h for SC in a cynomolgus monkey PK study respectively, longer than the above-mentioned recombinant IL15 molecules [20]. Similar to IAP0971, as previously reported, ALT-803 under SC administration has a greater tissue distribution and longer residence time in lymphoid tissues as compared with IV injection. Based on these results, we considered that SC injection is better for IAP0971 than IV infusion. However, the above-mentioned modifications still cannot maximize the effect of cytokines and improve the safety profile to achieve a decent therapeutic window. By adding a "navigator" of antibody to cytokines, immunocytokines with extended half-life and enhanced safety profile can synergize and specifically target cytokines to the tumor immune microenvironment, recruit, activate and reinvigorate immune cells, and achieve a much enhanced anti-tumor immunity [31–33]. As previously reported, one of the key reasons for acquired resistance of current PD(L)1-based immunotherapy is the development of exhausted T cells, with expression markers of PD1 + LAG3 + TIM3+ [34, 35]. Recently, several studies demonstrated that although the anti-PD1 therapy enriched terminally exhausted T cells, PD1/variant IL2 immunocytokines utilizing shared IL2/15R$\beta\gamma$ with IAP0971 generated and expanded effector memory T cells, and can reactivate PD1 + TIM3+ exhausted tumor-specific CD8 + T cells in the tumor [17, 36]. Previously, a novel PDL1/IL15 immunocytokine, named KD033 composed of a PDL1 antibody linked to the sushi-domain of the human IL15/IL15 receptor alpha complex has been described to achieve a robust and durable anti-tumor efficacy, and reduce toxicity associated with systemic IL15 exposure [37]. However, more recently, several studies have reported that cis-delivery of IL2 or IL15 to CD8 + T and NK cells in the tumor microenvironment through PD1-based immunocytokine shows better anti-tumor activities than PDL1-based immunocytokine, or no-targeting combination therapy [18, 36]. Even though we have validated that IAP0971 can simultaneously bind to PD1 and IL2/15R in both ELISA and cell-based FACS assays, we have not ruled out the possibility that IAP0971 may also act independently on IL15R or PD1 of effector cells.

In summary, we report here the development of IAP0971, a novel immunocytokine that binds specifically to PD1 and fuses IL15/IL15Rα complex, and preclinical characterization including pharmacology, pharmacodynamics, pharmacokinetics and toxicology studies. IAP0971 has demonstrated excellent anti-tumor efficacy and favorable safety profile, which has the potential to address primary non-responsiveness and acquired resistance of current immunotherapy. A Phase I/IIa clinical trial to evaluate the safety, tolerability and preliminary efficacy of IAP0971 in patients with advanced malignant tumors is on-going (NCT05396391).

SUPPLEMENTARY DATA

Supplementary Data are available at *ABT* Online.

DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article and in its online supplementary material.

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CONFLICT OF INTEREST STATEMENT

All authors are employees of SunHo (China) BioPharmaceutical Co., Ltd.

ETHICS AND CONSENT STATEMENT

Not applicable.

ANIMAL RESEARCH STATEMENT

All experiments were performed with the approval of the WuXi AppTec (Shanghai) Co., Ltd and WuXi AppTec

(Suzhou) Co., Ltd Institutional Animal Care and Use Committee (IACUC). Care was taken throughout the study to prevent any undue animal suffering.

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