Discovery of a multispecific CD73/PD-1 targeting Drug Fc-Conjugate (DFC), which improves tumor reduction compared to PD-1 monotherapy in a humanized mouse model

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BACKGROUND

The approval of PD-(L)1 axis inhibitors have firmly established immunotherapy as an effective cancer treatment option with reduced adverse effects compared with conventional chemotherapy[1]. However, while anti-PD-(L)1 therapy has demonstrated durable responses, only a small subset of patients respond. One mechanism of tumor escape from anti-PD-1 therapy is via the release of immunosuppressive metabolites such as adenosine. CD73, an extracellular enzyme expressed on immune cells and some tumors, catalyzes the rate limiting step in adenosine production, the conversion of AMP to adenosine[2] (**Figure 1A/B**). As a strategy to improve response rates, we developed a first-in-class multispecific CD73/PD-1 targeting DFC. Herein, we describe CD73/PD-1-001, a dual targeting DFC comprising a multivalent conjugate of a small molecule CD73 inhibitor stably linked to a proprietary human IgG1 Fc-fusion with a PD-1 inhibitor peptide (**Figure 2**). This multispecific DFC has the potential for differentiation and increased therapeutic efficacy compared to approved PD-(L)1 inhibitors.

Figure 1. Immune suppression by PD-1 and CD73 checkpoint pathways (**A**). Release of immune blockade by simultaneous targeting of PD-1 and CD73 (**B**).

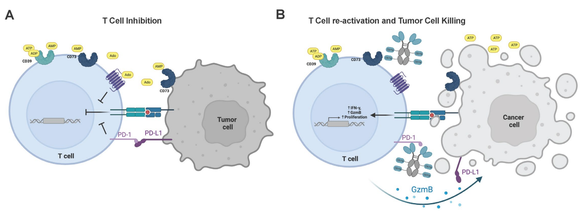
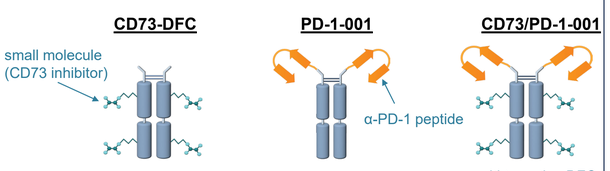


Figure 2. CD73 (left) and PD-1 (center) DFCs. Bispecific CD73/PD-1 DFC (rt).



METHODS

Binding of PD-1-001 and CD73/PD-1-001 to biotinylated hPD-L1 was measured by ELISA; test articles were also evaluated in a functional hPD-1 blockade assay (Promega #J1250). CD73 binding was evaluated by surface plasmon resonance (SPR) (ACROBiosystems). Efficacy was evaluated in transgenic mice expressing human PD-1 and PD-L1 with MC-38 (hPD-L1) tumors (genOway, France) (n=10). MC-38 (hPD-L1) cells were injected at a concentration of 10^6 cells mixed 1:1 with Matrigel (Corning). Treatment was initiated when tumor sizes were 25 – 75 mm^3. Test articles were dosed IP twice weekly for 3 weeks. As a comparator, a pembrolizumab biosimilar (Bio-X-Cell #SIM0010) was included. Tumor volumes were recorded, and statistical analysis was conducted by t-test (Mann-Whitney) or two-way ANOVA. *p≤0.05; **p≤0.01.

The PK of test articles was determined in BALB/c mice. DFCs were injected IP at 10 mg/kg (n=2) and plasma collected at various time points over one-week (168h). Plasma levels of DFCs were determined by indirect ELISA techniques with CD73 or hPD-1-001 capture, and a sandwich ELISA with human Fc capture. The activity of DFCs was determined in a Mixed Lymphocyte Reaction (MLR) assay. Briefly, human $CD14^+$ monocytes were differentiated into mature dendritic cells and incubated with $CD4^+$ cells from three separate donors in the presence/absence of AMP (300 μM). After four days supernatants were analyzed for cytokines (INF-γ, IL-2, TNF-α).

RESULTS

In vitro characterization of PD-1-001 and CD73/PD-1-001. The binding affinity of CD73/PD-1-001 for CD73 was determined by SPR using the Biacore 8k system (Cytiva). CD73/PD-1-001 was found to have a K_D of 0.66 nM confirming that it binds CD73 with high affinity. Multiple assays were used to confirm that PD-1-001 and CD73/PD-1-001 bind to hPD-1 with high affinity. Binding of both molecules to immobilized hPD-1 was measured by ELISA and found to be virtually identical with average EC_{50}'s from two assays of 0.055 and 0.064 nM for PD-1-001 and CD73/PD-1-001, respectively (**Figure 3**). A cell-based functional hPD-1/hPD-L1 signaling assay was used to compare CD73/PD-1-001 to approved PD-1 inhibitors. In this assay CD73/PD-1-001 was potent, demonstrating in vitro activity within ~2-fold of pembrolizumab, and ~5-fold of nivolumab biosimilars (**Table 1**).

Figure 3. Binding of DFCs to immobilized hPD-1.

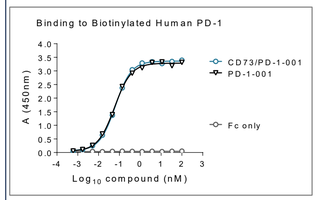


Table 1. hPD-1 Blockade assay

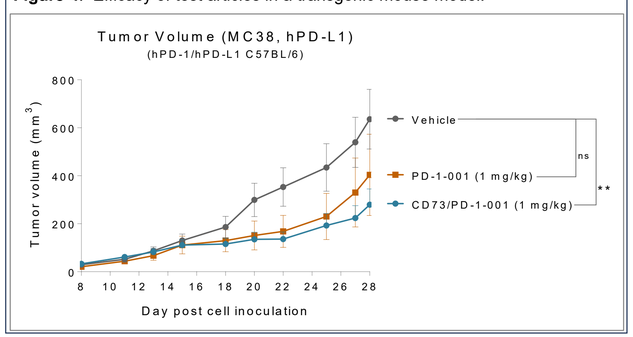
Test article	Target	EC_{50} (nM)
CD73/PD-1-001	CD73/PD-1	33.96
Pembrolizumab*	PD-1	18.10
Nivolumab*	PD-1	7.22

*biosimilar

In vivo characterization of PD-1-001 and CD73/PD-1-001 in a humanized mouse model. In transgenic mice expressing hPD-1/L1 a pembrolizumab biosimilar at 10 mg/kg had minimal effects on tumor growth (<10% TGI). Similarly, a CD73 only targeting DFC did not significantly reduce tumor growth (<20% TGI; Data not shown). In contrast, our proprietary anti-PD-1 inhibitor, PD-1-001, dosed at 10 mg/kg significantly reduced tumor growth (TGI of ~58%; p≤0.01). Nearly identical tumor volume reductions were also achieved with the multispecific CD73/PD-1-001 construct at 3 and 10 mg/kg (Data not shown).

In a follow-up study both DFCs were dose ranged at 1, 3, and 10 mg/kg. At the lowest test concentration (1 mg/kg) the DFCs began to differentiate, with CD73/PD-1-001 achieving a statistically significant reduction in tumor volume (p≤0.05) and a TGI of ~56% while PD-1-001 did not reach significance (**Figure 4**). At 3 and 10 mg/kg the dual inhibitor further increased TGI to 72.0 and 74.5% respectively. Similarly, PD-1-001 was significantly active at 3 and 10 mg/kg with TGI's of 71.4 and 80.8% respectively (Data not shown). The observation that PD-1-001 and CD73/PD-1-001 are differentiated at 1 mg/kg highlights the improved therapeutic potential of a dual CD73 and PD-1 targeting DFC.

Figure 4. Efficacy of test articles in a transgenic mouse model.



RESULTS

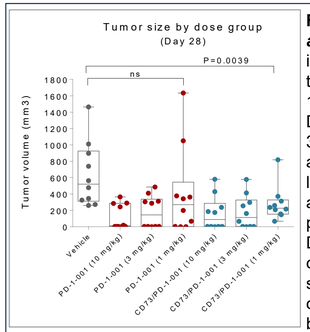
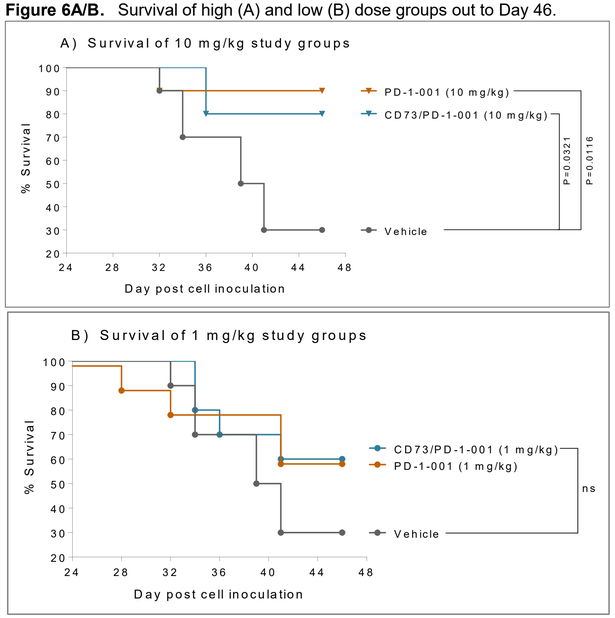


Figure 5. ***Box plot of individual animals on Day 28***. Analysis of individual animals on Day 28 supports the potency of PD-1-001 and CD73/PD-1-001 DFCs shown in **Figure 4**. Both DFCs demonstrate significant activity at 3 and 10 mg/kg relative to vehicle and are difficult to distinguish because the lack of a dose response suggest they are at/near the maximum achievable potency (**Figure 5**). At 1 mg/kg the dual DFC demonstrates a much tighter clustering of tumor volumes which is significant, while PD-1-001 is not at this concentration, further demonstrating a benefit to the dual inhibitor (**Figure 5**).

PD-1-001 and CD73/PD-1-001 prolong survival compared to vehicle treated animals through Day 46. Using survival for 46 days as the end point, both PD-1-001 and CD73/PD-1-001 demonstrated a significant survival advantage (90 and 80% respectively) when dosed at 10 mg/kg (**Figure 6A**). At the lowest test concentration of 1 mg/kg both DFCs doubled the survival rate compared to vehicle, although this was not statistically significant (**Figure 6B**). Importantly, this study was not designed as a survival study and all animals received their last dose on Day 22, so although both demonstrate potent in vivo efficacy, differentiating DFCs with this end point is hindered.

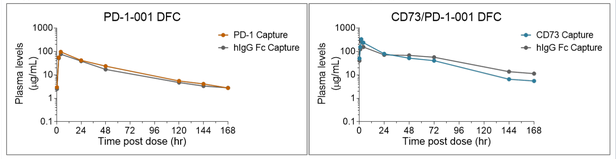
Figure 6A/B. Survival of high (A) and low (B) dose groups out to Day 46.



PK of PD-1-001 and CD73/PD-1-001 in BALB/c mice. To confirm that differences in TGI between the DFCs was not the result of a difference in exposure, a PK study was conducted. Using hPD-1, CD73, or Fc capture methods, both DFCs were determined to have similar PK profiles (**Figure 7**).

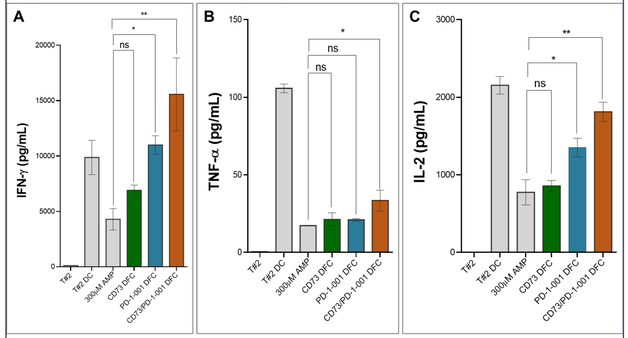
RESULTS

Figure 7. PK of DFCs in BALB/c mice (IP, 10 mg/kg).



Activity of DFCs in a MLR assay to confirm release of checkpoint blockades. To elucidate the mechanism behind in vivo efficacy, an initial PD-1 driven one-way MLR was run using mature dendritic cells, and CD4+ T-cells from three donors. Both DFCs demonstrated ~2-fold or more induction of INF-γ, TNF-α, and IL-2 relative to an irrelevant mAb which was on par for the increase seen with a pembrolizumab biosimilar (Data not shown). To determine the benefits of combining CD73 and PD-1 in a single DFC, 300 μM AMP was added to the assay to establish a second inhibitory checkpoint. Each cytokine showed a significant increase relative to AMP only suppressed cells (**Figure 8**, panels A-C). Collectively these assays confirm the activity of both CD73 and PD-1 inhibitors comprising the dual DFC.

Figure 8. One-way mixed lymphocyte assay (MLR) (*P<0.0332, **P< 0.0021)



SUMMARY

PD-1-001, a proprietary IgG1 Fc-anti-PD-1 fusion peptide construct, was modified by the addition of a stably-conjugated CD73 small molecule inhibitor, creating CD73/PD-1-001, a dual targeting DFC against two important immune checkpoints. The dual DFC was found to bind its target ligands with high affinity, which translated to exceptional potency in a humanized mouse model with TGIs of ~56 and ~75% at 1 and 10 mg/kg doses, respectively. Both constructs demonstrated stable PK over a week in BALB/c mice. A MLR assay with and without AMP suppression confirmed the functionality of both CD73 and PD-1 moieties for the dual inhibitor as evidenced by increased production of important pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-2). CD73/PD-1-001 is a potent therapeutic targeting both the clinically relevant PD-(L)1 and adenosine axes. Optimization is underway to nominate a potential development candidate.

DISCLOSURE & REFERENCES

All authors are shareholders & employees of Cidara Therapeutics.

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[1]Franzin et. al., 2020; PMID: 33162990

[2]Xia et. al., 2023; PMID: 36859386