Preclinical toxicology and pharmacology for the 4-IBB/HER2 bispecific PRS-343: A first-in-class costimulatory T cell engaging
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**Background**

4-IBB (CD137) is a key costimulatory immunoreceptor and a highly promising therapeutic target in cancer. To overcome toxicity and efficacy limitations of current 4-IBB-targeting antibodies, we have developed a novel, 4-IBB/HER2 bispecific based on AntirAd Technology. We have previously reported on the generation and characterization of PRS-343 with regard to preclinical proof-of-concept and basic drug-like properties (1). Here, we describe the preclinical dataset supporting initiation of a first-in-patient trial.

The pharmacology of PRS-343 is investigated by ex vivo assays based on mixed culture of human PBMC and tumor cell lines. The assays are used to determine the cytokine profile of T cell stimulated by PRS-343 induced 4-IBB clustering. Using a set of immortal cancer cell lines and primary cells spanning a range of HER2 surface copy numbers, we identify the threshold required to elicit a costimulatory response, and a lower bound to ensure that costimulation can be reliably achieved. The risk of PRS-343-mediated, systemic 4-IBB activation and concomitant toxicity is investigated in a cytokine release assay model of human peripheral blood mononuclear xenograft-to-host disease (xDH). HER2-mediated toxicity is studied in a GLP-compliant, repeat-dose toxicity study in cynomolgus monkeys.

The combined dataset provides an overview on the pharmacology, mode of action and safety profile of PRS-343.

**Concept: tumor-specific and tumor-localized costimulatory activation of T cells**

![Diagram of T cell activation](image)

**PRS-343 design, target binding and activity in reporter and T cell costimulation assay**

<table>
<thead>
<tr>
<th>A</th>
<th>Anti-4-IBB Antibody</th>
<th>B</th>
<th>PRS-343 Design</th>
<th>C</th>
<th>PRS-343 Design (DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>HER2 ELISA</td>
<td>E</td>
<td>E-IBB ELISA</td>
<td>F</td>
<td>Dual binding ELISA</td>
</tr>
</tbody>
</table>

**PRS-343 induced cytokine release in the absence of T cell receptor stimulation is negligible**

![Diagram of cytokine release](image)

**PRS-343 costimulated T cells express IL-2, GM-CSF, IFNγ and TNFα**

- T cells were co-cultured with HER2+ NCi-N87 cells and PRS-343.
- Supernatant concentrations were determined for a panel of cytokines.
- Cytokines prominently induced by PRS-343-mediated costimulation were GM-CSF, IL-2, IFNγ and TNFα.
- These cytokines may serve as pharmacodynamic biomarkers in clinical studies.

**PRS-343-mediated T cell costimulation requires supraphysiologic HER2 levels**

- The costimulatory T cell activation assay was performed for a series of tumor cell lines and primary cells covering a wide range of HER2 positivity.
- An anti-4-IBB benchmark mAb was used as a positive control.
- Response specificity was controlled with an excess of trastuzumab.
- The series of experiments showed (a) relative costimulatory activity on HER2 levels corresponding to 4% of SKBR-3 (HER2+2) and 3% of SKBR-3 (HER2-0) did not costimulate in the physiological HER2 expression range (24% of SKBR-3), (b) variable donor effect results in the intermediate range (3% to 5%), (c) Costimulatory activity was observed in SUM1525 and T47D-1 cells described as restricted to at least one costimulatory targeted therapy (4-6).

**PRS-343 is well tolerated in repeat-dose cynomolgus monkey toxicity study**

- The safety of PRS-343 was investigated in a GLP-compliant cynomolgus monkey study. PRS-343 was given in weekly doses of 0, 10 and 100mg/kg over 4 weeks as an intravenous or subcutaneous administration dosing schedule study.
- Delayed onsets or reversibility of toxicity was studied in recovery groups (0 and 120mg/kg) and also at both doses tested, with significant results.
- TK analysis demonstrated full, dose-proportional exposure at both dose levels, with a terminal half-life of 5-6 days.

**Conclusion**

- **PRS-343 is a 4-IBB/HER2 bispecific based on the genetic fusion of a high-affinity 4-IBB binding Anticalin and modified trastuzumab**
- **The presented preclinical pharmacology and toxicology studies confirm previous results (1) and support that PRS-343 elicits its costimulatory effects specifically on T cells also co-receiving a TCR signal and strictly localized to HER2-positive tumors:**
  - **PRS-343-mediated 4-IBB activation requires supraphysiologic HER2 levels**
  - **PRS-343 costimulation leads to increased production of multiple pro-inflammatory cytokines associated with anti-tumor immune response**
  - **The risk of systemic 4-IBB activation is low on negligible cytokine release in the absence of primary T cell receptor engagement**
- This is supported by a humanized mouse toxicology study, where PRS-343 avoids the systemic peripheral activation of CD8+ T cells observed with a benchmark 4-IBB antibody.
- A GLP-compliant cynomolgus monkey toxicity study demonstrates the safety profile of PRS-343 in a repeat-dose cynomolgus monkey study. The reported data support evaluation of PRS-343 in a Phase I study in patients with HER2-positive advanced or metastatic solid tumors.

**Table 1: Study Design**

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Dose Level (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>PRS-343</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>PRS-343</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 2: Number of Animals per Group**

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Number of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>3 (2 females, 1 male)</td>
</tr>
<tr>
<td>B</td>
<td>PRS-343</td>
<td>3 (2 females, 1 male)</td>
</tr>
<tr>
<td>C</td>
<td>PRS-343</td>
<td>3 (2 females, 1 male)</td>
</tr>
</tbody>
</table>

**Figure 1**: Cytokine induced by human T cells co-stimulated by PRS-343 in the presence of HER2-positive NCi-N87 cells in T-cell co-stimulation assay. Cytokine levels in the culture supernatants were measured by an electrochemiluminescence (ECL) immunoassay.

**Figure 2**: Cytokine release assay with PRS-343, PRS-343 incubated with HER2+ positive tumor cells in a T cell costimulation assay. Cytokine levels in the culture supernatants were measured by an electrochemiluminescence (ECL) immunoassay.

**Figure 3**: Costimulatory 4-IBB T cell engagement by PRS-343. Within a patient’s tumor, tumor-specific T cells are engaged with tumor cells by the costimulatory bispecific PRS-343 which simultaneously binds the tumor target HER2 and the immune receptor 4-IBB. The engagement of 4-IBB provides a local co-stimulatory signal to the T cell, further enhancing the T cell receptor engagement. The resulting activation of the T cell is dependent on the receptor engagement, which is expected to be tolerogenic. In PRS-343, two 4-IBB binding domains on the Fab construct are critical for activation of 4-IBB in the absence of target-positive cells, and healthy, functional human T cells may be activated by the Fab construct due to the presence of a primary, TCR-mediated signal.

**Figure 4**: Cytokine release assay with PRS-343. T cells were isolated from the blood of healthy donors and incubated for 72 hours with PRS-343 after air dried, in affordable, or T cell-coated. Four concentrations of PRS-343 in a volume of 100µl were tested in each condition as indicated in the figure. The anti-4-IBB costimulation benchmark (OET1) at all different concentrations served as the positive control, and an IgG4 isotype antibody was the negative control. Supernatant levels of two cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12P70, GM-CSF, IFNγ and TNFα) were analyzed. The figure shows the average response for four donors that displayed a significant response to OET1, and for a selection of the most relevant cytokines.

**Figure 5**: PRS-343 costimulatory dependence on target cell HER2 level. Tumor or primary cells of different HER2 positivity were subjected to a T cell costimulatory activation assay using IL-2 expression and cytokine production. For each cell type, the cells were performed with at least two independent donors. The half-maximum costimulatory dose of T cells to be costimulated in the presence of costimulatory anti-CD3 was reported. Figure ETOH used IgG4 isotype or transport activity. Anti-4-IBB benchmark cell was the positive control. The experiment was performed also in the presence of an excess of trastuzumab to inhibit the binding of PRS-343 to the SKBR-3 cells. The best statistical significance of a donor vs. control level was reported for tumor cell lines and primary cells (p<0.005 (**), p<0.05 (*) or p>0.05 (>). Values of p>0.05 were considered not statistically significant (ns). Bottom line: Cytokine expression levels were plotted for each tumor cell line and primary cell type on a logarithmic scale.

**Figure 6**: PRS-343 activity in xenograft mouse models. Tumor-bearing mice were injected with HER2-positive SK-OV-3 cell line and human PBMC. A) Median of tumor growth. B) Frequency of CD8+ T cells determined by intracellular cytokine staining of tumor cells only. One out of three independent experiments is presented. Data are all expressed as means ± SEM. For further experimental details.