**Glypican-3 (GPC3) and NKp46 directed FLEX-NK™ cell engager antibody (CYT-303) distributes to tumors and shows dose-dependent tumor growth inhibition in a hepatocellular carcinoma (HCC) mouse model**

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**Abstract**

BACKGROUND: GPC3 is an oncofetal antigen that is highly expressed in HCC while it is hardly expressed in adult normal tissues except placenta. CYT-303 is a multifunctional bispecific NK cell engager built on our FlexiNK™ scaffold, which engages NK cells through MHCII and targets GPC3 expressed on tumor cells. Previously, we have reported that CYT-303 showed in vitro redirected Hep3B tumor cell cytotoxicity as well as in vivo Hep3B tumor growth inhibition with peripheral blood NK cells (PBNK) and our NK cells derived from iPSC (iNK), respectively. Here, we further characterized the in vivo CYT-303 dose-response for tumor growth inhibition and its influence on NK cell trafficking and distribution in the blood and the tumor. METHODS: CYT-303 pharmacokinetics were evaluated in PBNK injected HGS-HL35 mouse bearing subcutaneous Hep3B tumors. PBNK circulation in blood was analyzed by flow-cytometry and CYT-303 distribution in blood and tumor by PK immun unstained. Blood alpha fetoprotein (AFP) was measured by Immunoassay. RESULTS: Similar CYT-303 PK profile was observed in HGS tumor bearing mice compared to non-tumor bearing mice. In the Hep3B tumor model in PBNK or iNK injected Hep3B tumor bearing mice, CYT-303 showed dose-dependent tumor growth inhibition associated with control Hep3B treated mice. Consistently with CYT-303 dose-dependent tumor growth inhibition, dose-dependent increases in CYT-303 concentration were observed in the tumor. Blood NK cell count in CYT-303 treated animals were significantly lower compared to iNK treated control mice suggesting CYT-303 may facilitate NK cell trafficking from blood into the tumor. Blood alpha fetoprotein (AFP), a biomarker in HCC, decreased in CYT-303 tumor growth inhibition. CONCLUSIONS: Pharmacologically active CYT-303 dosages were identified and CYT-303 distribution to the tumor was demonstrated suggesting NK trafficking to the tumor.

**Figure 1: CYT-303 mediated NK cell activation in HCC**

- Targeting structure allows higher reactivity for GPC3 tumor and NKp46 NK cell targets, improved affinity and specificity
- Tomato linker allows for simultaneous binding to tumor target and NK cells facilitating tumor uptake
- FLEX-NK™ construct enhances NK cell function against target cells

**Figure 2: Immunophenotypic profile of iNK cells and PBNK cells**

- iNK and PBNK expanded similarly with feeder cells were harvested and immunophenotyped using above indicated directly conjugated antibody and isotype control antibodies. Mean fluorescence intensity (MFI) and percent positive cell staining with each antibody are shown.

**Figure 3: CYT-303 pharmacokinetic profile in HCC tumor bearing mice**

- Mean fluorescence intensity (MFI) and percent positive cell staining with each antibody are shown.

**Figure 4: CYT-303 combination with PBNK and iNK cells show dose-dependent tumor growth inhibition in HCC tumor bearing mice**

- PBNK and iNK cell numbers were analyzed by flow cytometry and are consistent with the bell-shaped dose-response observed in the HCC tumor models.

**Figure 5: CYT-303 treatment showed reductions in blood PBNK cells suggesting trafficking to tumors**

- CYT-303 or control IgG1 was administered (ip) by intraperitoneal injections at the above indicated doses to HGS-HL35 mouse bearing Hep3B subcutaneous tumor that were intratumorally injected on day 0 with 5.0 x 10^6 PBNK cells. Blood was collected at the indicated times, measured using a CYT-303 target capture and detected PK immun unstained, and analyzed using WinMDI non-compartmental analysis.

**Figure 6: CYT-303 levels in plasma & tumor provide a correlation between efficacious concentrations in blood and tumor**

- CYT-303 plasma and intratumoral levels were measured 24 h post-CYT-303 dose in the HGS-HL35 Hep3B tumor model using PK immun unstained.

**Figure 7: CYT-303 binding and cytotoxicity dose-responses with PBK and iNK cells are consistent with the dose-responses observed in the HCC tumor models**

- The bell-shaped in vitro dose-responses observed in vitro for CYT-303 binding to PBK and Hep3B tumors and CYT-303 driven PBK cytotoxicity of Hep3B are consistent with the bell-shaped dose-response observed with CYT-303 in the PBNK injected HCC tumor model.

- The linear dose-response observed in vitro for CYT-303 driven Hep3B cytotoxicity of Hep3B are consistent with the bell-shaped dose-response observed with CYT-303 in the Hep3B injected HCC tumor model.

- The lower PBK binding and Hep3B cytotoxicity at CYT-303 doses is likely driven by monocytes binding due to excess antibody or dose resulting in lower antibody binding and signaling of the agents resulting in a bell-shaped dose-response in vitro and in vivo- Presence effect.

**Figure 8: Alpha Fetoprotein level reductions following CYT-303 treatment in HCC tumor models**

- CYT-303 pharmacologically active iNK cell dosed reductions in alpha fetoprotein (AFP) biomarker compared to control treated animals in both PBK and iNK injected models.

- AFP reductions with CYT-303 in the HCC model is statistically significant compared to PBS treated mice.

- AFP immunoassay was performed according manufactures instructions (Invitrogen).

**Conclusions**

- CYT-303 showed dose-dependent HCC tumor growth inhibition in PBK and iNK cell injected HCC tumor models.
- Dose-dependent increases in CYT-303 concentrations in tumor and blood were observed in the PBK injected HCC tumor model showing the potential of CYT-303 to penetrate solid tumors.
- CYT-303 treated animals showed significant decreases in blood PBK cells suggesting CYT-303 may facilitate trafficking of these cells from blood to the tumor.
- Bell shaped dose-response observed with CYT-303 monotherapy in the PBK injected HCC tumor model is consistent with CYT-303 in vitro studies showing similar dose responses for PBK and iNK tumor binding and tumor cytolytcs.
- The linear dose-response observed with CYT-303 combination therapy with iNK cells in the HCC tumor model is consistent with the in vitro linear dose-response observed with iNK combination for cytolytcs of HCC tumors.
- CYT-303 treatment resulted in reductions in blood AFP levels in both the PBK and iNK injected HCC tumor models showing the utility of this biomarker for CYT-303 clinical studies.
- These CYT-303 preclinical proof-of-principle studies support clinical development of CYT-303 in HCC.