Increasing immune cell infiltration in hepatocellular carcinoma tumors using a novel GPC3-targeting aptamer conjugate

Christina McGuire, Aaron Ball, Justin Sonberg, Jin Yuan, Yuxun Wang, Kristin Thompson, Shuhao Zhu

Guardian Therapeutics, Lexington, MA

Abstract

Glypican 3 (GPC3) is a cell membrane-bound heparin sulfate proteoglycan, expressed at the cell surface of many cancer types. It has emerged as a leading target for hepatocellular carcinoma treatment due to its overexpression in aberrant liver cells and low expression in healthy adult tissues. One of the major challenges in treating hepatocellular carcinoma with current immunotherapies is turning cold tumors into immunogenic ones. Unmethylated CpG oligodeoxynucleotides (ODNs) are known immune stimulators, however their use in combination therapy for cancer treatment is limited due to the requirement of intra tumoral injections to prevent systemic toxicity. Here, we describe a unique GPC3-specific aptamer, able to target GPC3-expressing cancer cells *in vivo* as a means to deliver the immunostimulatory CpG 7909. Systemic administration through intra peritoneal (i.p.) injection of the GPC3 aptamer-CpG conjugate (GRX51) decreases Hep3B tumor volume growth in a Balb/c nude mouse xenograft model by ~30-50% depending on treatment schedule, while also increasing immune cell infiltration. Final tumor volumes of GRX51-treated animals are consistent with tumor volumes of animals treated with the standard of care compound, sorafenib tosylate. Due to its GPC3-targeting capacity, systemic dosing of GRX51 (15 mg/kg) is less toxic than systemic administration with equimolar amounts of CpG 7909 alone (5 mg/kg), with a comparable effect on tumor shrinkage. While both GRX51 and CpG 7909 induce immune cell recruitment, GRX51 treated mice had a final immune:tumor cell ratio of 3.1 ± 1.9, while CpG 7909-treated animals had a ratio of 0.6 ± 0.4 relative to the vehicle control after a 7-day on/7-day off daily dosing schedule. Using the Hep3B xenograft in CD34+ humanized mice, we also showed GRX51 primes tumors for combination treatments with FDA approved checkpoint inhibitors. Mice were treated with GRX51 (7.5 mg/kg daily i.p. dose, 11 days), the PD-1R inhibitor pembrolizumab (10 mg/kg bi-weekly i.p., 11 days), or a combination of the two drugs. The GRX51 treatment alone outperformed pembrolizumab alone, reducing tumor size by 33% and 18% respectively. Notably, the combination of GRX51 with pembrolizumab reduced tumor size by 63%. Together, our data show that GRX51 is a novel GPC3-targeting molecule capable of reducing tumor size and priming GPC3-positive tumors for combination immunotherapy, without inducing major systemic toxicity with i.p. injection.

Introduction

- GPC3 is a biomarker for hepatocellular carcinoma (HCC).[1]
- Unmethylated CpG oligodeoxynucleotides (CpG-ODNs) are immune stimulators that are used as vaccine adjuvants, but have limited therapeutic value in treating cancer because they require intra-tumoral injections.[2,3]
- CpG 7909 is a B-Type CpG-ODN TLR9 agonist that stimulates B cells and dendritic cells.

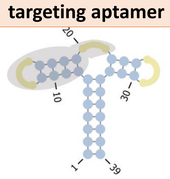
Can we increase immune cell infiltration into tumors without causing systemic toxicity by targeting CpG ODNs to HCC using an aptamer against GPC3?

Generating GRX51

Using SELEX against recombinant GPC3-Fc protein, we isolated and optimized a very nuclease resistant GPC3 binding aptamer.

- ❖ See AACR Annual Meeting 2021 Abstract #934.

Optimized GPC3-targeting aptamer

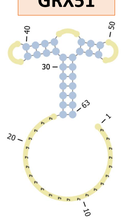


CpG ODN 7909 (GRX30) is a class B CpG that stimulates TLR9.

GRX30 (CpG ODN 7909)

GRX30 was added to the 5' end of the optimized GPC3 aptamer, generating GRX51.

GRX51



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Results

GRX51 binds to human GPC3 protein

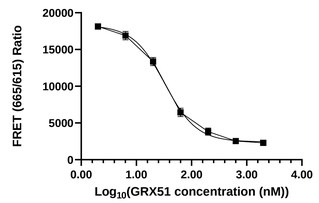

Fig. 1 FRET competition assay to test the ability of GRX51 to bind human GPC3 protein. Fluorescence signal was quenched as GRX51 displaced the GPC3-binding aptamer (non CpG-conjugated) from GPC3 protein. Curve fitting analysis determined an IC50 of 32.7 nM.

GRX51 stimulates IL-6 release *in vitro* and *in vivo* to a similar degree as CpG alone

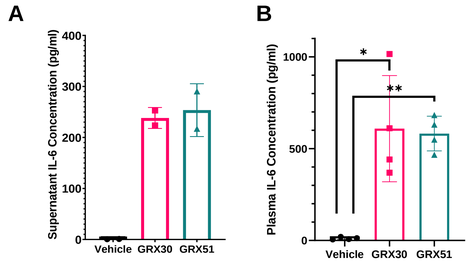

Fig. 2 A) 24-hour treatment of isolated human PBMCs with 100 nM of GRX51 or GRX30. Supernatant IL-6 levels were determined using a quantitative ELISA assay. B) BALB/c nude mice were treated with equimolar amounts of GRX30 or GRX51. Mice were given 5 mg/kg GRX30 or 14 mg/kg GRX51 by s.c. injection, and plasma was collected 2 hours later. Plasma IL-6 levels were determined using a quantitative ELISA assay. Error bars represent S.D. Significance between groups was determined using an unpaired t test, * represents $p < 0.05$ and ** represents $p < 0.01$.

Systemic dosing with GRX51 in BALB/c nude mice is less toxic than with GRX30 with a comparable effect on final Hep3B tumor weight

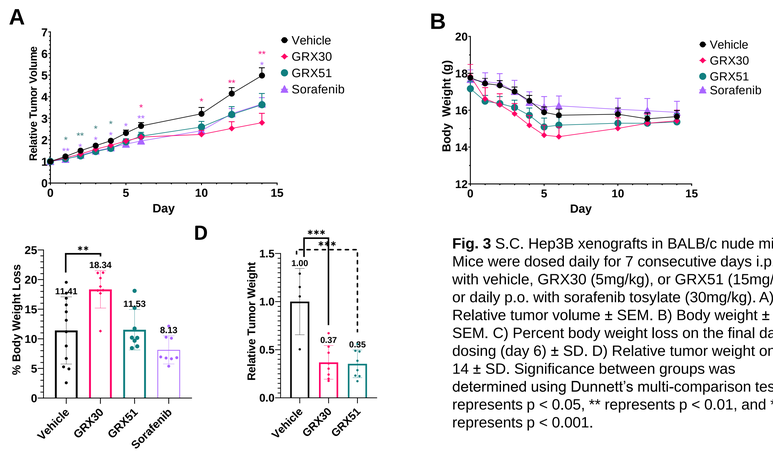

Fig. 3 S.C. Hep3B xenografts in BALB/c nude mice. Mice were dosed daily for 7 consecutive days i.p. with vehicle, GRX30 (5mg/kg), or GRX51 (15mg/kg), or daily p.o. with sorafenib tosylate (30mg/kg). A) Relative tumor volume ± SEM. B) Body weight ± SEM. C) Percent body weight loss on the final day of dosing (day 6) ± SD. D) Relative tumor weight on day 14 ± SD. Significance between groups was determined using Dunnett's multi-comparison test, * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$.

Systemic dosing of GRX51 in BALB/c nude mice increases the intra-tumoral immune cell population

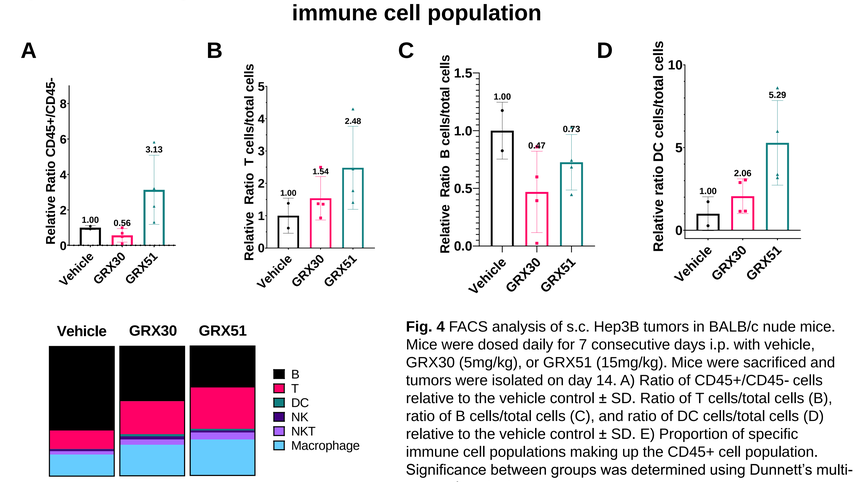

Fig. 4 FACS analysis of s.c. Hep3B tumors in BALB/c nude mice. Mice were dosed daily for 7 consecutive days i.p. with vehicle, GRX30 (5mg/kg), or GRX51 (15mg/kg). Mice were sacrificed and tumors were isolated on day 14. A) Ratio of CD45+/CD45- cells relative to the vehicle control ± SD. Ratio of T cells/total cells (B), ratio of B cells/total cells (C), and ratio of DC cells/total cells (D) relative to the vehicle control ± SD. E) Proportion of specific immune cell populations making up the CD45+ cell population. Significance between groups was determined using Dunnett's multi-comparison test.

Combination treatment with GRX51 and pembrolizumab decreases Hep3B tumor volume more than individual treatments in CD34+ humanized mice

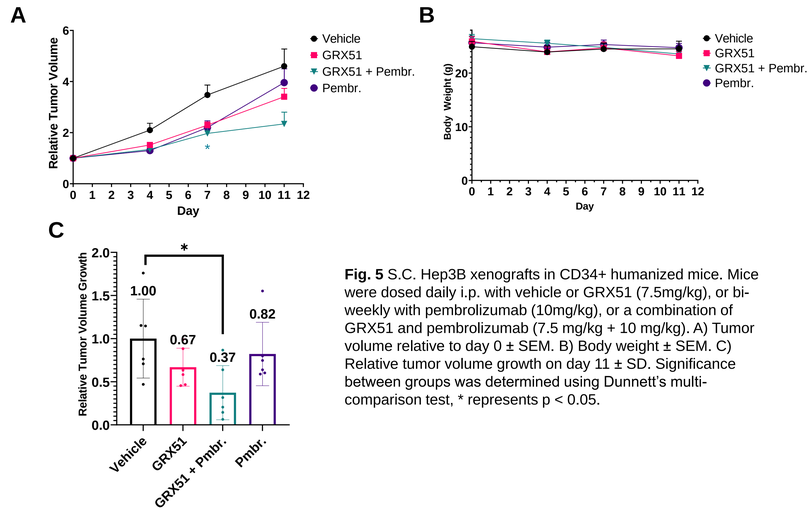

Fig. 5 S.C. Hep3B xenografts in CD34+ humanized mice. Mice were dosed daily i.p. with vehicle or GRX51 (7.5mg/kg), or bi-weekly with pembrolizumab (10mg/kg), or a combination of GRX51 and pembrolizumab (7.5 mg/kg + 10 mg/kg). A) Tumor volume relative to day 0 ± SEM. B) Body weight ± SEM. C) Relative tumor volume growth on day 11 ± SD. Significance between groups was determined using Dunnett's multi-comparison test, * represents $p < 0.05$.

GRX51 treatment induces IL-6 release, but not recruitment of human immune cells into the Hep3B tumor in CD34+ humanized mice

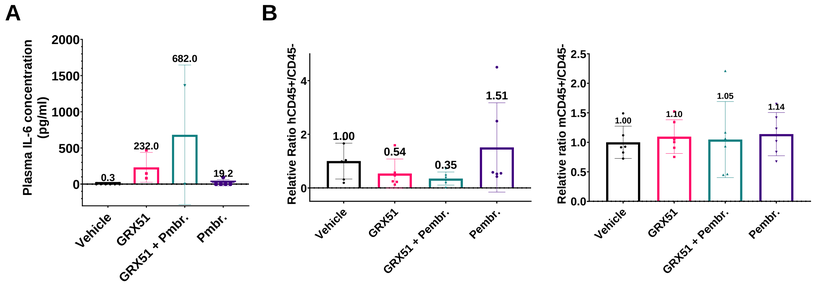

Fig. 6 A) Plasma collected from CD34+ humanized mice 2 hours after final dose with vehicle, GRX51 (7.5 mg/kg, QD 14 days i.p.), pembrolizumab (10 mg/kg, BIW 14 days i.v.), or a combination of GRX51 and pembrolizumab (7.5 mg/kg + 10 mg/kg, 14 days). IL-6 levels were measured using a quantitative ELISA assay. Results show mean ± SD. B) FACS analysis of s.c. Hep3B tumors in CD34+ humanized mice. Mice were dosed daily for 21 consecutive days with vehicle or GRX51 (7.5mg/kg, i.p.), or bi-weekly with pembrolizumab (10mg/kg, i.v.), or a combination of GRX51 and pembrolizumab (7.5 mg/kg + 10 mg/kg). Mice were sacrificed and tumors were isolated on day 21. Ratio of human (hCD45+) or mouse (mCD45+) CD45+/CD45- cells relative to the vehicle control ± SD. Significance between groups was determined using Dunnett's multi-comparison test.

Discussion

Here we describe the use of a novel anti-GPC3 aptamer (see abstract #934) to target the immunostimulatory CpG ODN 7909 to hepatocellular carcinoma. The use of CpG ODN's in cancer treatment is complicated by the fact that they require intra-tumoral injection to properly stimulate and recruit immune cells to the tumor[3]. We show systemic dosing of the aptamer-CPG conjugate, GRX51, binds GPC3 (Fig. 1) and stimulates IL-6 release *in vivo* and *in vitro* to a similar degree as CpG alone (GRX30; Fig 2). We hypothesize that the GPC3 aptamer portion of GRX51 allows for localization of the compound intra-tumoraly, thus reducing the systemic toxicity seen with GRX30 (Fig. 3C) after systemic i.p. dosing in a Hep3B xenograft model in BALB/c nude mice. Both GRX51 and GRX30 inhibited tumor growth to a similar degree (Figs 3A, 3D) however only GRX51 increases immune cell recruitment to the tumor (Fig. 4). Specifically, GRX51 increased the number of T and DC cells within the tumor relative to the total cell population (Fig. 4B-E). While anti-tumor effects were also seen after daily GRX51 treatment in a CD34+ humanized mouse model, combination treatment with pembrolizumab (a PD-1R inhibitor) reduced tumor size to a larger degree than either drug treatment alone, indicating an additive effect of the two immunostimulatory molecules (Fig. 5). Both GRX51 and the combination treatment with pembrolizumab increased the plasma IL-6 concentration in these mice, however there was no increase in the number of mouse immune cells recruited to the tumor, and a decrease in the number of human immune cells recruited to the tumor in both treatment groups relative to the vehicle control (Fig. 6). Together this data suggest that targeting of CpG to a tumor using a GPC3 aptamer rather than through intra-tumoral dosing is an effective strategy to reduce tumor size, with less toxicity and more intra-tumor immune stimulation than CpG alone in hepatocellular carcinoma. Furthermore, GRX51 is a prime candidate for combination therapy with checkpoint inhibitors in GPC3+ cancers.

References

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2. Klinman, D. M. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol* **4**, 249–259 (2004).
3. Lou, Y. *et al.* Antitumor Activity Mediated by CpG: The Route of Administration is Critical. *Journal of Immunotherapy* **34**, 279–288 (2011).