A half-life extended, tumor-activated IL-12 increased the infiltration of effector immune cells into the tumor microenvironment and demonstrated anti-tumor activity in syngeneic mouse models

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Introduction

Interleukin-12 (IL-12) is a proinflammatory cytokine that bridges innate and adaptive immunity via induction of T helper 1 differentiation and promotes cytolytic activity of natural killer cells, as well as effector T cells. IL-12 has demonstrated potent anti-tumor activity in syngeneic mouse models and promising anti-tumor activity in humans. However, clinical development of IL-12 has been limited by severe (grade 5) systemic toxicities. XTX301 was engineered as a potent, half-life extended and masked IL-12 designed to overcome toxicity and improve the therapeutic index of IL-12. The masking domain of XTX301 was designed to pharmacologically inactivate IL-12 in non-tumor tissue. The cleavage site was designed to be cleaved by proteases that are enriched in the tumor microenvironment (TME) enabling the generation of an active IL-12 moiety in the TME.

Design of XTX301, a tumor-activated IL-12

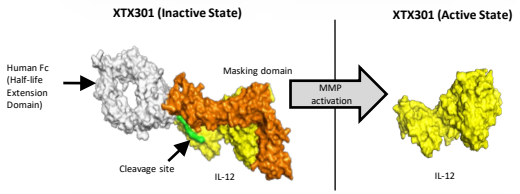


Figure 1: The masking domain of XTX301 was designed to pharmacologically inactivate IL-12 in non-tumor tissue and render an active IL-12 moiety upon cleavage by proteases that are enriched in the tumor microenvironment. MMP: matrix metalloproteinase

Masking of XTX301 IL-12 activity and reactivation by MMPs in vitro

XTX301 is pharmacologically active upon proteolytic activation in cell-based assays

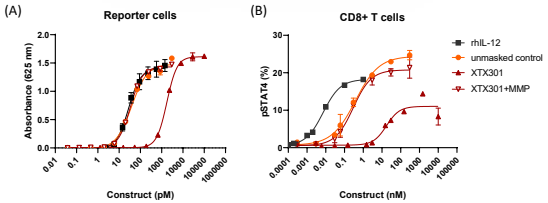


Figure 2: (A) IL-12 activity was measured with an IL-12 reporter gene cell line, which is engineered to express colorimetric reporter upon IL-12-mediated STAT4 activation. Cells incubated with XTX301 demonstrated reduced reporter activity compared to cells treated with rhIL-12 or unmasked control molecule. Upon MMP activation, XTX301 demonstrated full potency. (B) Primary human peripheral blood mononuclear cells (PBMCs) were preactivated with PMA/ionomycin and then incubated with varying doses of rhIL-12 and test articles for 30 minutes. Flow cytometry was used to identify CD8+ T cells and evaluate STAT4 phosphorylation. XTX301 resulted in attenuated STAT4 phosphorylation compared to rhIL-12 and unmasked control. Upon MMP activation, XTX301 resulted in a similar STAT4 phosphorylation as unmasked control.

Anti-tumor activity of mXTX301 in vivo

mXTX301 demonstrated anti-tumor activity in hot and cold syngeneic mouse tumor models

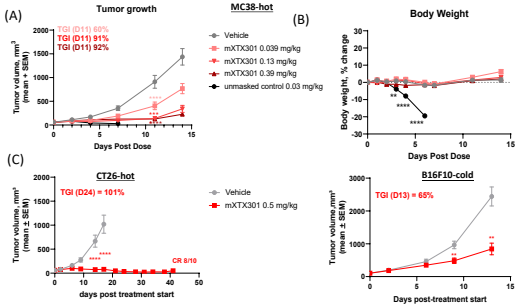


Figure 3: mXTX301 is a murine surrogate for XTX301. mXTX301 was made for *in vivo* studies since human IL-12 does not bind to mouse IL-12 receptors. mXTX301 demonstrates anti-tumor activity in MC38, CT26 and B16F10 syngeneic tumor models. (A) C57BL/6 mice were implanted subcutaneously with MC38 tumor cells and received a single intravenous injection of mXTX301, unmasked control, or vehicle (PBS) at indicated dose levels (N=12). Tumor and body weight measurements were taken two/three times a week. Data represent mean ± SEM (Standard error of the mean). Tumor volume was assessed by a two-way ANOVA with Bonferroni's post-hoc pairwise comparison test compared to vehicle (PBS) treated animals.**** P <0.0001 compared to vehicle-treated animals on Day 11. The tumor growth changes are displayed until Day 14 as this is when 75% of vehicle-treated animals were alive (N=9). (B) Body weight data are displayed until Day 14 when 75% of the vehicle-treated group were alive (N=9). (C) Animals were implanted subcutaneously with CT26 or B16F10 tumor cells and received a single intravenous injection of mXTX301 at 0.5 mg/kg. Tumor measurements were taken two/three times a week (N=8-10 per group). Tumor growth data are presented as mean for tumor volume ± SEM. Two-way ANOVA followed by Bonferroni post-hoc test (**P<0.005; ****P<0.0001). TGI: tumor growth inhibition; CR: complete regression

mXTX301 in vivo activity is dependent on tumor associated proteases in the MC38 mouse tumor model

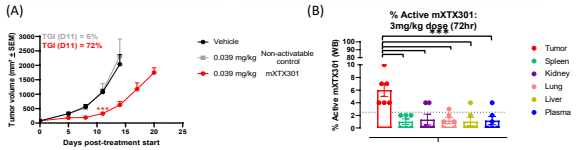


Figure 4: Tumor-associated protease-dependent activation of mXTX301 in vivo. (A) C57BL/6 mice were implanted subcutaneously with MC38 tumor cells and received a single intravenous injection of mXTX301, or non-activable control lacking protease cleavage site or vehicle at indicated dose levels. Data represent mean ± SEM. Tumor measurements were assessed by a two-way ANOVA with Bonferroni's post hoc pairwise comparison test compared to vehicle (PBS) treated animals.*** P <0.0005 compared to vehicle-treated animals on Day 11. The tumor growth inhibition was calculated at day 14 as this is when 86% of vehicle-treated animals were alive (N=6). TGI: tumor growth inhibition (B) Measurement of % of active mXTX301 in the tumor, peripheral organs, and plasma, in the MC38 syngeneic tumor model. MC38 mice were dosed with 3 mg/kg mXTX301. After 72h, mice were sacrificed, blood was collected and the tumor, lung, spleen, kidney, and liver were resected. Tissues were lysed in RIPA buffer. mXTX301 in tissue lysate was immunoprecipitated using anti-human IgG and the percentage of active mXTX301 was determined by fluorescent triplex WB (cut-off 2.5%). Statistical comparisons were performed using Dunnett ordinary one-way ANOVA versus tumor, where ***P < 0.001.

Mechanism of action of mXTX301 in vivo

mXTX301 was effectively masked in the periphery in the MC38 mouse tumor model

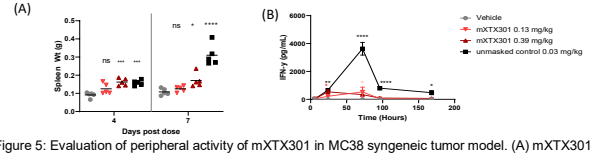


Figure 5: Evaluation of peripheral activity of mXTX301 in MC38 syngeneic tumor model. (A) mXTX301 at 0.13 mg/kg did not impact spleen weight at days 4 and 7. Unmasked control at 0.03 mg/kg resulted in significant splenomegaly on days 4 and 7. Statistical comparisons were performed using a one-way ANOVA versus control, where *P < 0.05, ***P < 0.001, ****P < 0.0001. (B) mXTX301 at doses 0.13, and 0.39 mg/kg resulted in an increase of plasma IFNγ with a peak at 72 and 24 hours, respectively. Compared to masked mXTX301, unmasked control resulted in 7-10-times higher IFNγ response at 72 hours. The level of cytokine remained high at 96 and 168 hrs. Statistical comparisons were performed using a one-way ANOVA versus control, where *P < 0.05, **P < 0.005, ****P < 0.0001.

mXTX301 induced tumor selective pharmacodynamic effects in the MC38 syngeneic model

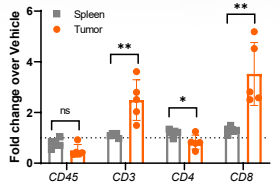


Figure 6: MC38 tumor bearing mice (N= 5 per group) were treated with a single IV dose of mXTX301 at 0.39 mg/kg or vehicle, and immune cells were phenotyped using flow cytometry. The number of cells for each immune phenotype was calculated per gram of tissue, and the ratio of cells after mXTX301 treatment to after vehicle treatment is presented as mean ± standard deviation. Differences in the ratio of each cell type in spleen and tumor were assessed by an unpaired t test. *P < 0.05, **P < 0.005.

mXTX301 induced pro-inflammatory gene programs and broad remodeling of the TME

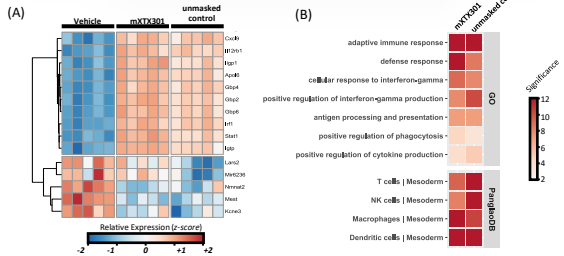
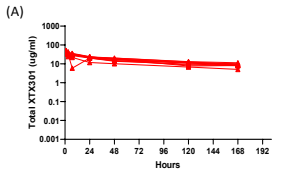


Figure 7: Comprehensive evaluation of mXTX301 induced gene expression changes in MC38 tumors by bulk RNA sequencing. (A) Heatmap showing relative expression of top mXTX301 differentially expressed genes (N = 10 upregulated and N = 5 downregulated; by P-value). Rows (genes) were clustered according to Euclidean distance. Keys above each column (sample) indicate treatment group (vehicle, mXTX301 [0.39 mg/kg], or unmasked control [0.03 mg/kg]). Box color tracks with z-score-transformed relative expression of each gene across samples (blue, under-expression compared to the mean; red, over-expression compared to the mean). (B) GO: Gene ontology; NK: natural killer cells. Heatmap summarizing results of a pathway enrichment analysis where each box indicates the enrichment significance (-log10 Fisher P-value) of a gene set of interest (rows) in the top 250 most significantly upregulated genes (by P-value) computed by comparing either mXTX301 or unmasked control to vehicle at Day 7 (columns). Color intensity tracks with significance.

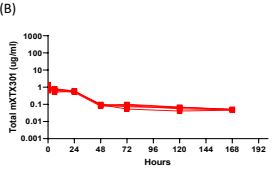
Pharmacokinetics and therapeutic window of XTX301 in preclinical studies

XTX301 pharmacokinetics (PK) in non-human primates at highest non-severely toxic dose (HNSTD) and mean PK parameters



Dose (mg/kg)	C_{max} (µg/mL)	AUC_{last} (hr*µg/mL)	$T_{1/2}$ (days)
2	43	2540	4.1

mXTX301 PK in mice at dose with anti-tumor activity and mean PK parameters



Dose (mg/kg)	C_{max} (µg/mL)	AUC_{last} (hr*µg/mL)	$T_{1/2}$ (days)
0.13	1	38	2.4

XTX301 has the potential for a broad therapeutic index

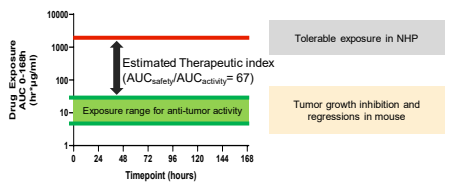


Figure 8: Preclinical data support the potential for a broad therapeutic index for XTX301. The area under-the-curve parameter was determined from 0 to 168 hours for (A) concentration versus time profiles from nonhuman primates (N=5/sex) administered the highest non-severely toxic dose of XTX301, 2.0 mg/kg QWx4, and (B) concentration versus time profiles from MC38 tumor-bearing mice (N=5) administered an efficacious dose of mXTX301, 0.13 mg/kg. (C) A large exposure difference in the group mean $AUC_{0\text{-}168hr}$ between HNSTD in non-human primates and dose with anti-tumor activity in mice supports the potential for a broad therapeutic index for XTX301.

Conclusions

mXTX301, a mouse surrogate of XTX301, a half-life extended, masked tumor-activated IL-12 molecule, demonstrated anti-tumor activity in syngeneic mouse models. Unlike the non-activatable control molecule, mXTX301 demonstrated protease dependent activity and significantly inhibited tumor growth in mice at 0.039 mg/kg. mXTX301 was more highly cleaved in mouse tumors compared to organs and plasma, demonstrating tumor-selective activation. mXTX301 had limited impact on spleen weight, peripheral immune cells and plasma IFNγ levels demonstrating effective peripheral masking. In mouse model tumors, mXTX301 increased CD8 T cell infiltration and induced proinflammatory gene signatures. In cynomolgus monkeys, the HNSTD was 2 mg/kg dosed weekly for 4 doses. These preclinical data support the potential for a broad therapeutic index for XTX301. XTX301 will be evaluated in a Phase I clinical trial (NCT05684965).