SITC 2025

Abstract # 861

Sequential Administration of WTX-124 and mWTX-330, IL-2 and IL-12 INDUKINE™ Molecules, Enhanced Antitumor Activity in Mice Bearing Poorly Immunogenic EMT6 Tumors Without Systemic Toxicity

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Werewolf THERAPEUTICS

Building a Novel Class of Conditionally Activated Immunotherapies for Patients with Cancer

BACKGROUND

Combination Strategies to Enhance Antitumor Activity in IO resistant Tumors

Though proinflammatory cytokines such as Interleukin-2 (IL-2) and Interleukin-12 (IL-12) have been shown to promote initiation and/or amplification of adaptive antitumor immune responses, routine use of the approved high dose IL-2 regimen and clinical development of recombinant human IL-12 have been impeded by severe toxicities. We previously reported clinical data from novel tumor-activated INDUKINE molecules for native IL-2 (WTX-124) and IL-12 (WTX-330) demonstrating that each cytokine prodrug showed encouraging monotherapy clinical activity and tolerability in the outpatient setting with evidence of T cell activation and expansion in on-treatment tumor biopsies (NCT05660384, NCT5678998). Here we used IL-2 and IL-12 INDUKINE molecules to explore whether the mechanisms of action of IL-12 (e.g., generation of *de novo* antitumor responses through IFNγ-enhanced antigen presentation) and IL-2 (e.g., expansion and enhanced effector function of CD8 T cells) can be safely combined in syngeneic tumor models to improve antitumor activity, especially in poorly immunogenic tumors. We found that concurrent administration of WTX-124 and WTX-330 improved antitumor activity over either cytokine alone but was poorly tolerated. Therefore, we explored whether sequential administration of mouse surrogate WTX-330 (mWTX-330) and WTX-124 could similarly enhance antitumor activity but with less toxicity.

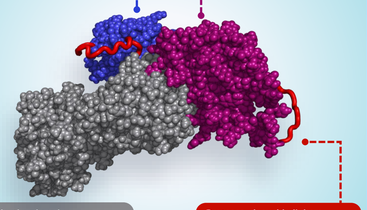
Results

In the EMT6 model, which is resistant to WTX-124, a single dose of mWTX-330 followed by WTX-124 up to one week later was well tolerated and demonstrated enhanced antitumor activity compared to vehicle, WTX-124 alone or a single dose of mWTX-330. Multiplex immunofluorescence analysis of tumors revealed that mWTX-330 initially increased infiltration of CD103+ cross presenting dendritic cells (DC) and then subsequently increased the frequency of polyfunctional CD8 T cells and caused an M2 to M1 shift in tumor associated macrophage (TAM) phenotypes. Sequential dosing of WTX-124 further increased the number of polyfunctional CD8 T cells and M1 macrophages. At the end of the study, ELISpot analysis demonstrated that mWTX-330 had primed tumor specific immune responses, which were further amplified by WTX-124. We conclude that sequential administration of mWTX-330 and WTX-124 takes advantage of the complementary mechanisms of action of native IL-12 and IL-2, eliciting marked antitumor activity with improved tolerability compared to concurrent dosing regimens. We believe that this approach may be directly translatable to the clinical setting.

Key Features of INDUKINE Molecules

Half-life extension domain
- Confers high tumor exposure while also extending the dosing interval

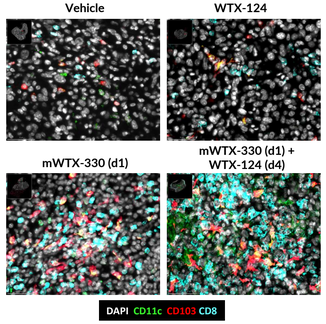
Cytokine domain
- Full potency cytokines
  - Wild-type IL-2
  - Wild-type IL-12



Inactivation domain
- Blocks activation of cytokine receptors
- Limits peripheral target-mediated disposition
- Designed to limit peripheral toxicity

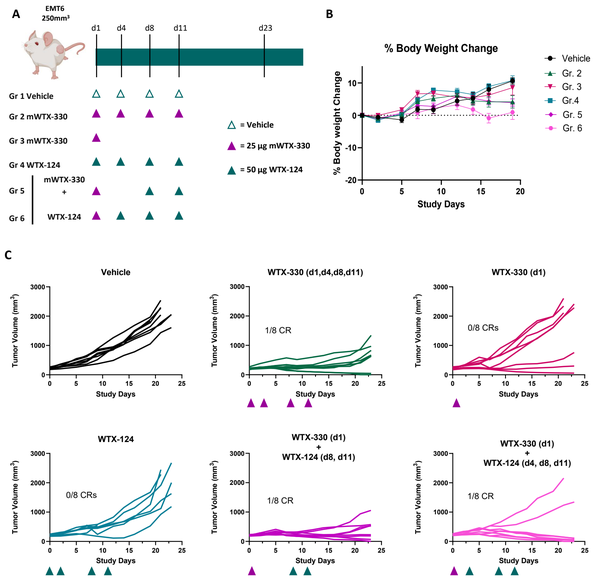
Protease-cleavable linkers
- Novel protease substrates with optimal tumor selectivity
- Efficient cleavage in numerous human solid tumors *in vitro* with minimal cleavage by non-tumor tissues

mWTX-330 Recruits Cross-Presenting Dendritic Cells to the Tumor Microenvironment



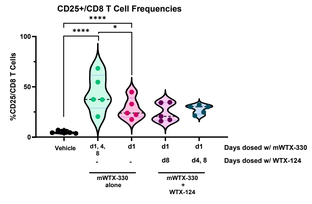
EMT6 tumor bearing mice (n=3 per group) were treated with a 25 μg sub-efficacious dose of mouse surrogate WTX-330, 50 μg WTX-124 or in sequence. Tumors were harvested at Day 6 then fixed in formalin before being embedded in paraffin, sectioned, and dewaxed and antigen retrieved. Tumors were stained and imaged using a Lunaphore COMET™ multiplex immunofluorescence platform. Timepoints at which test articles were dosed are in parentheses. Representative images show staining for DAPI, CD11c, CD103 and CD8.

Sequenced Dosing of mWTX-330 and WTX-124 Resulted in Robust Antitumor Activity in Poorly Immunogenic EMT6 Tumors



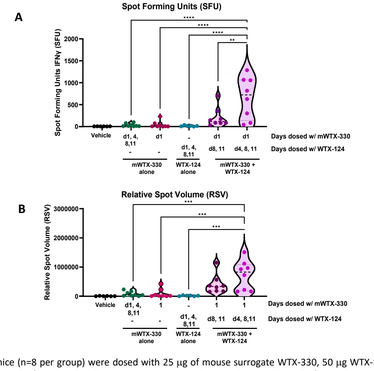
EMT6 tumor bearing mice (n=8 per group) were dosed with 25 μg of mouse surrogate WTX-330 alone or in combination with 50 μg WTX-124 at indicated times points. Tumors and body weight were measured twice weekly. **(A)** schematic depicting study design **(B)** mean body weight change **(C)** individual spider plots. CR=complete regression

mWTX-330 Primed Intratumoral CD8 T Cells to Respond to IL-2 Through CD25 Upregulation



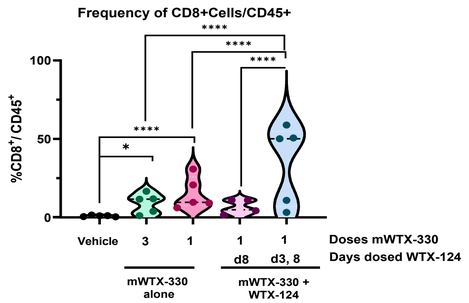
EMT6 tumor bearing mice (n=5 per group) were dosed with 50 μg of mouse surrogate WTX-330, 50 μg of WTX-124 or in combination. Tumors were harvested at day 9, dissociated and stained for flow cytometry. Shown here is the frequency of CD25+ CD8+ TILs. Two way ANOVA analysis was performed, and significance is reported as follows *p<0.05, and *****p<0.0001

WTX-124 Amplified mWTX-330-Driven *de novo* Antitumor Responses



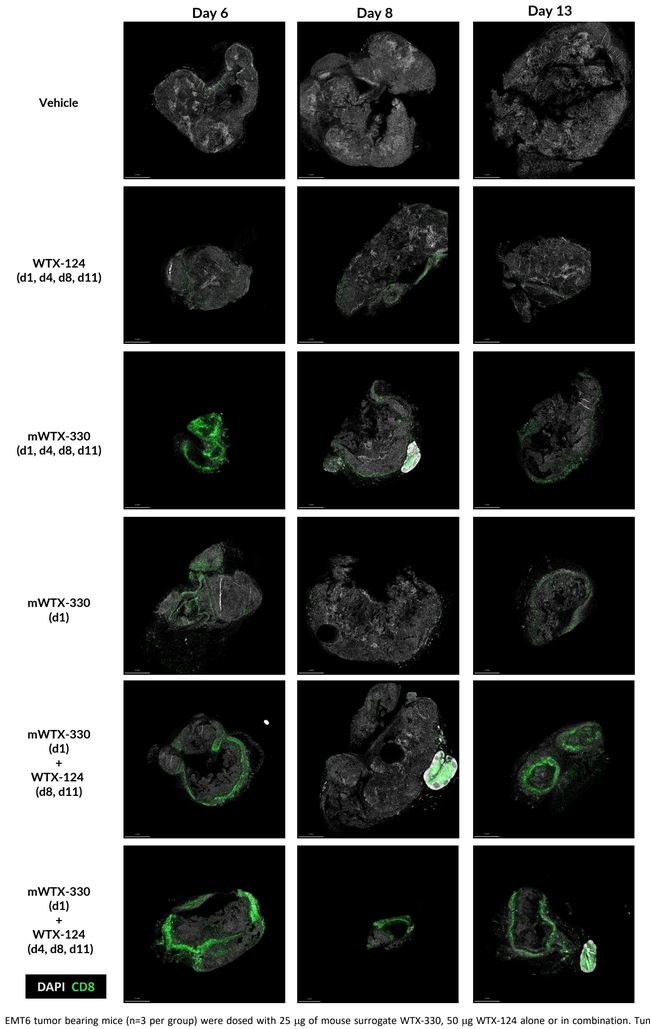
EMT6 tumor bearing mice (n=8 per group) were dosed with 25 μg of mouse surrogate WTX-330, 50 μg WTX-124 or in combination. Spleens were harvested at the end of study, plated in an IFNγ ELISpot with or without EMT6 cells for 48 hours, imaged on Mabtech IRIS™ for quantification. **(A)** Spot forming units (SFU) **(B)** Relative spot volume (RSV). One way ANOVA was performed, and significance is reported as follows. **p<0.005, ***P <0.0005 *****p<0.0001

WTX-124 Increased Infiltration of CD8 T Cells after Priming with mWTX-330



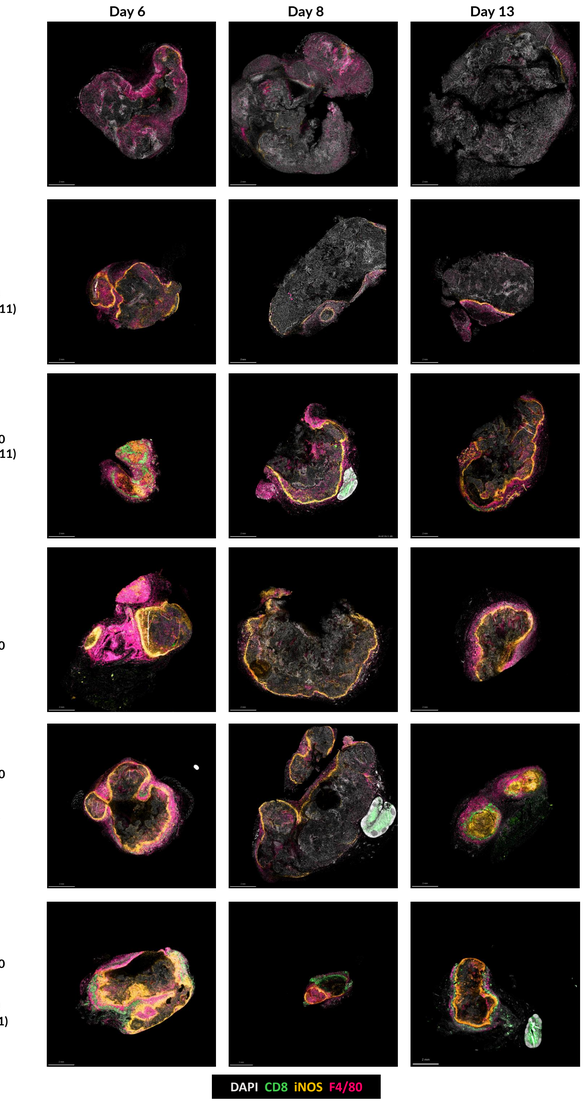
EMT6 tumor bearing mice (n=5 per group) were dosed with 50 μg of mouse surrogate WTX-330 alone or in combination with 50 μg WTX-124 at indicated times points. Tumors were harvested at day 9, dissociated and stained for flow cytometry. Shown here is the frequency of CD8+/CD45+TILs. Two way ANOVA analysis was performed, and significance is reported as follows *p=0.05, and *****p<0.0001

WTX-124 Amplified and Sustained Infiltration of CD8 T Cells after Priming with mWTX-330



EMT6 tumor bearing mice (n=3 per group) were dosed with 25 μg of mouse surrogate WTX-330, 50 μg WTX-124 alone or in combination. Tumors were harvested at indicated timepoints then fixed in formalin before being embedded in paraffin, sectioned, dewaxed and antigen retrieved. Tumors were stained and imaged using a Lunaphore COMET™ multiplex immunofluorescence platform. Representative images show DAPI and CD8 staining.

WTX-124 Increased M1 Macrophages in the TME After Priming with mWTX-330



EMT6 tumor bearing mice (n=3 per group) were dosed with 25 μg of mouse surrogate WTX-330 alone or in combination with 50 μg WTX-124. Tumors were harvested at indicated timepoints, then fixed in formalin before being embedded in paraffin, sectioned, dewaxed and antigen retrieved. Tumors were stained and imaged using a Lunaphore COMET™ multiplex immunofluorescence platform. Representative image showing DAPI, CD8, F4/80, and iNOS.

SUMMARY and CONCLUSIONS

- WTX-330 drives de novo tumor-specific immune responses, tumor infiltration of CD8 T cells and CD25 upregulation, creating a TME responsive to WTX-124
- Sequenced combination (WTX-330→WTX-124) drives inflammatory macrophage recruitment into the tumor microenvironment which may contribute to the combination's antitumor activity
- Sequenced administration of mWTX-330 followed by WTX-124 is well tolerated in mice and has robust antitumor activity
- These data indicate that WTX-330 can prime the tumor microenvironment to respond to WTX-124 and potentially be used to sensitize less immunogenic tumor types to WTX-124
- We believe that the sequencing of WTX-330 followed by WTX-124 may be directly translatable to the clinical setting

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