Generation of Novel Potent Human TREX1 Inhibitors Facilitated by Crystallography

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Abstract #1636

TEMPEST THERAPEUTICS

ABSTRACT

Background

STING is an innate immune sensor critical for the development of immunity. Tumor cells can inactivate the STING pathway to avoid immune recognition, emphasizing its importance in generating tumor-specific immunity. Broad activation of STING in advanced cancers may be required to initiate CD8$^+$ T cell priming against unique antigenic repertoires among distinct metastases and to reverse an immune-suppressive TME. Due to its ubiquitous expression, systemic delivery of untargeted STING agonists may not achieve a therapeutic index. In contrast, expression of the cytosolic DNAse 3' repair exonuclease (TREX1) is upregulated in tumor cells in response to genomic instability, inflammatory stimuli, and DNA replication, providing an opportunity for selective activation of the STING pathway. In addition to TREX1's role in modulating cGAS/STING signaling, its interaction with DNA replication enzymes that generate immunogenic DNA waste highlights a facet of TREX1 biology that may inform clinical development of targeted inhibitors. Treatment with TREX1 inhibitors could therefore enable broad targeting of metastatic tumors.

Methods

Using a structure-based drug design strategy, we designed and optimized small-molecule inhibitors of TREX1 with drug-like physicochemical properties that were profiled in biochemical and cell-based assays. X-ray crystallography studies, thermal shift, and biochemical assays were employed to determine mechanism of action. We evaluated the in vivo profile of select compounds.

Results

Inhibitors of TREX1 with < 100 μM potency were optimized into a series with nanomolar potency against purified, recombinant murine and human TREX1 protein in biochemical assays. Inhibitors had similar IC50 values against TREX1 nuclease in an intact cell-based assay. Finally, TSA results demonstrated that compound interaction requires magnesium. High-resolution co-crystal structures of inhibitor-bound TREX1 present a rationale for mouse- and human-specific interactions and confirms MOA predicted by earlier modeling efforts. Lead compounds demonstrated good bioavailability and achieved exposures necessary for target engagement in mouse models and resulted in tumor growth inhibition when combined with low-dose doxorubicin.

Conclusions

We present the identification and characterization of a potential first-in-class TREX1 inhibitor with nanomolar potency against human and mouse TREX1. Treatment with TREX1 inhibitors conferred profound anti-tumor activity when combined with DNA-damaging agents. Here we demonstrate that targeting TREX1 can specifically and locally engage the STING pathway in the tumor microenvironment, enhance tumor-specific immunity, and provide therapeutic benefit.

BACKGROUND

STING is a genetically validated drug target in humans

- Critical pivot point in immune decisions
- Tumors modulate STING signaling to avoid immune recognition
- Profound human disease results from STING pathway mutations

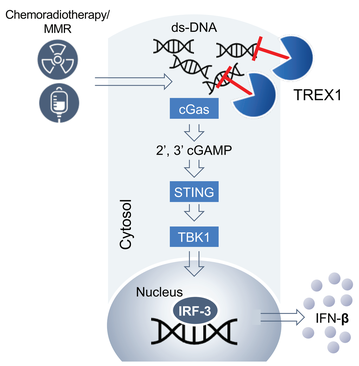
TREX1 controls activation of STING pathway

- Upregulated in TME due to:
  - Genetic instability
  - Inflammation
  - DNA replication
  - Therapeutic intervention such as cytotoxic therapy and radiation

TREX1 inhibitor enables systemic dosing to selectively activate STING in the TME

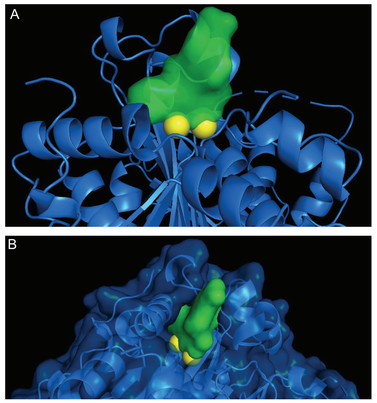
- Approach to broadly activate innate immune signaling in metastatic disease

TREX1 controls activation of STING



METHODS

Figure 1: X-ray Co-Crystal Structure Guided Drug Design



C

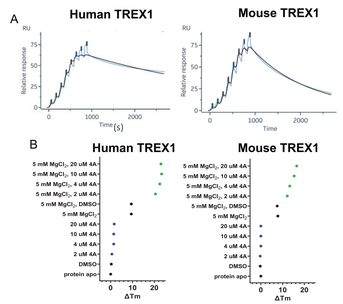
		Lead Molecule
Biochemical Potency	Human Picogreen dsDNA Assay (nM)	≤ 10 nM IC_{50}
Cellular Potency (IFNb)	THP1, HCT116, or other (nM)	< 1 μM EC_{50}
Selectivity	Toxicity/Specificity	CC_{50} > EC_{50} in cellular assays
Physicochemical Properties	MW / cLogD	< 500 / < 4
Pharmacokinetics	Rodent PK	% F > 30 Low-Moderate Clearance

Developing a first-in-class TREX1 inhibitor using co-crystal structures of inhibitor-bound TREX1

A) First X-ray co-crystal structure of human TREX1 (blue) in complex with inhibitor 4A (green) confirms Mg^{2+} -dependent mechanism of inhibition. The two catalytic Mg^{2+} ions in the active site are shown in yellow. Crystallized human TREX1 is a human to mouse chimera containing the following point mutations: A5T, P8H, P10H (ref). The previously published human TREX1 crystal structures lack the two catalytic Mg^{2+} ions in the active site. B) Surface representation of 4A-bound TREX1 co-crystal structure shows inhibitor in active site binding pocket. C) Human TREX1 inhibitor Target Candidate Profile (TCP) shows desirable properties for potent and selective TREX1 inhibitors

RESULTS

Figure 2: Biophysical Characterization of Recombinant TREX1 with Compound 4A by SPR and TSA

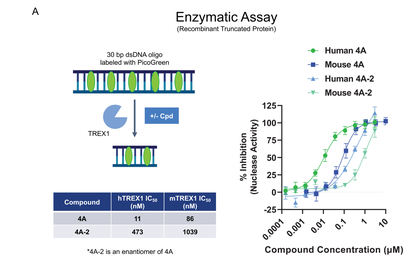


Species	K_D (nM)	Residence time (min)
Human	78	67
Mouse	414	21

Surface plasmon resonance (SPR) and thermal shift analysis (TSA) for compound 4A with human and mouse TREX1(2-242) recombinant protein

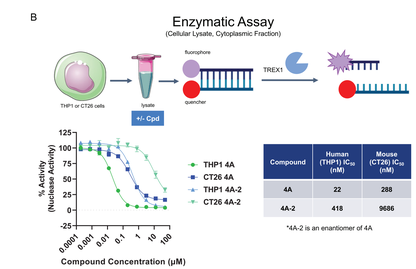
A) Single-cycle kinetics SPR analysis with site-specifically biotinylated human and mouse TREX1(2-242) shows nanomolar binding affinity for compound 4A B) Thermal shift analysis with (green) and without (blue) $MgCl_2$ confirms a Mg^{2+} ion-dependent mode of binding for compound 4A. Additionally, $MgCl_2$ enhances the thermal stability of TREX1 recombinant protein

Figure 3. Compound 4A and 4A-2 Activity in Biochemical Nuclease Assays



Compound	hTREX1 IC_{50} (nM)	mTREX1 IC_{50} (nM)
4A	11	86
4A-2	473	1039

*4A-2 is an enantiomer of 4A



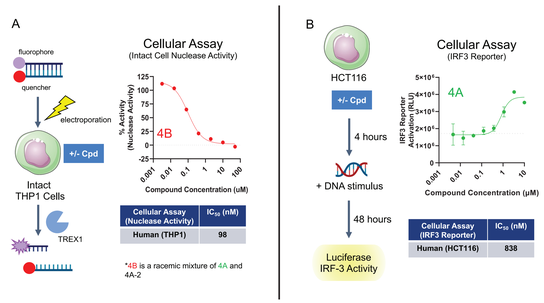
Compound	Human (THP1) IC_{50} (nM)	Mouse (CT26) IC_{50} (nM)
4A	22	288
4A-2	418	9686

*4A-2 is an enantiomer of 4A

Compound 4A inhibits both human and mouse TREX1 nuclease activity more potently than its enantiomer (4A-2) in biochemical activity assays.

A) Compound 4A inhibits both human and mouse recombinant TREX1 nuclease activity. Truncated TREX1 recombinant protein encompassing the catalytic domain (residues 2-242) was incubated with compound 4A and a double-stranded DNA substrate was added to initiate the nuclease reaction. Double-stranded DNA remaining at specific time points were quantified by PicoGreen fluorescence. B) Compound 4A inhibits TREX1 nuclease activity from both CT26 and THP1 cytoplasmic lysates. Cytoplasmic lysates from CT26 or THP1 cells expressing endogenous, full-length TREX1 were incubated with compound 4A. A double-stranded DNA substrate covalently-modified with a 5' fluorophore on the sense strand and 3' fluorescence quencher on the antisense strand was added to initiate the nuclease reaction. TREX1 nuclease activity can be quantified by measuring fluorescence intensity over time. Negligible non-TREX1 nuclease activity was detected from CT26 and THP1 TREX1 knock out lysates (data not shown). Data is shown as mean +/- SD.

Figure 4. Compound 4B and 4A Activity in Intact Whole Cell Assays



Cellular Assay (Nuclease Activity)	IC_{50} (nM)
Human (THP1)	98

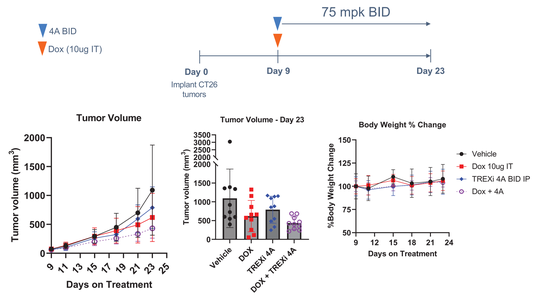
*4B is a racemic mixture of 4A and 4A-2

Cellular Assay (IRF3 Reporter)	IC_{50} (nM)
Human (HCT116)	838

Compound 4B and 4A inhibit nuclease activity and induce STING/IRF3 signaling in intact cells, respectively.

A) Compound 4B inhibits TREX1 nuclease activity in intact THP1 cells. A double-stranded DNA substrate covalently-modified with a 5' fluorophore on the sense strand and 3' fluorescence quencher on the antisense strand was introduced into THP1 cells by electroporation. Electroporated cells containing the fluorophore-conjugated DNA substrate were rested for 10 minutes, followed by treatment with compound 4B. TREX1 nuclease activity can be quantified by measuring fluorescence intensity over time. Negligible non-TREX1 nuclease activity was detected from THP1 TREX1 knock out cells (data not shown). Compound 4B is a racemic mixture of compound 4A and 4A-2. B) Compound 4A increases STING activation in DNA-stimulated HCT116 IRF3 reporter cells. HCT116 cells were treated with compound 4A for 4 hours at 37˚C, followed by transfection with BstNI-digested pBR322. DNA-stimulated cells were then incubated at 37˚C for an additional 48 hours before monitoring for IRF3 reporter luciferase activity. Luciferase reporter activity with compound 4A treatment was compared to DNA-stimulation alone (dashed line). No IRF3 reporter activity above DNA alone was detected in HCT116 TREX1 knock out reporter cells with compound 4A treatment (data not shown) confirming that enhanced IRF3 signaling after compound 4A treatment is a TREX1-dependent effect. Data is shown as mean +/- SD.

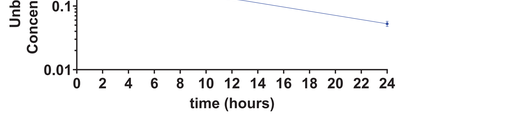
Figure 5. Compound 4A Demonstrates Anti-Tumor Activity in Combination with Doxorubicin



Compound 4A inhibition of TREX1 activity results in anti-tumor activity when combined with low-doses of doxorubicin (Dox).

A) Groups of 10 Balb/c mice were implanted with CT26 tumor cells on D0. On D9, tumors were given 10 ug of doxorubicin intratumorally and treated with compound 4A at 75 MPK BID for 14 days. Tumor growth and body weight were measured every 3 days until D23. Body weights demonstrated no additive toxicity with doxorubicin. Data is shown as mean +/- SD.

Figure 6. Compound 4A Pharmacokinetic Profile



Unbound tumor concentrations of compound 4A are above its in vitro IC50 value up to 4 hours after dosing.

Compound 4A was administered to C57BL/6 mice at 60 mpk IP BID for 14 days. On day 14 tumor samples were collected at 15 min, 4 h, and 24 h. Concentrations of compound 4A was measured by LC-MS/MS. Data is shown as mean +/- SD.

CONCLUSIONS

Maximizing the therapeutic index of STING activation for cancer therapy may require simultaneous targeting of all metastatic tumors while still limiting non-selective systemic STING activation

- Designed a novel series of first-in-class TREX1-specific inhibitors with nanomolar potency in biochemical assays
- Solved the first human TREX1 co-crystal structure in complex with the catalytic Mg^{2+} ions and a potent TREX1 inhibitor
- Developed multiple biochemical, biophysical, and cell-based assays to profile TREX1 inhibitors
- Demonstrated TREX1-inhibition mediated STING activation in vitro and anti-tumor activity in vivo
- Studies with lead series compounds underway to characterize both STING-dependent and STING-independent tumor efficacy activities

REFERENCE

1. Zhou, Wen, Desmond Richmond-Buccola, Qiannan Wang, and Philip J. Kranzusch. "Structural Basis of Human TREX1 DNA Degradation and Autoimmune Disease." Nature Communications 13, no. 1 (December 2022): 4277. https://doi.org/10.1038/s41467-022-32055-z.