Transcriptional profiling of *Bicycle*® tumor-targeted CD137 agonist-treated mouse tumors revealed an early and rapid activation of myeloid cells followed by infiltration of cytotoxic T cells into the tumor

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#1356

ABSTRACT

CD137 (4-1BB) is an immune costimulatory receptor that has been recognized for its potential as an immunotherapy drug target in cancer alongside checkpoint inhibitors [1-2]. We have developed a new class of modular synthetic drugs, termed *Bicycle*® tumor-targeted immune cell agonists (*Bicycle*® TICAs), which are multifunctional molecules composed of constrained bicyclic peptides [3]. The first molecule of this class, BT7480, a Nectin-4-dependent CD137 agonist, entered clinical trials in 2021. Preclinical data demonstrates that BT7480 induces highly potent, tumor localized CD137 agonism leading to tumor regressions and immunogenic memory in a syngeneic mouse model [4]. In this work, we sought to understand the effect of our *Bicycle* tumor-targeted immune cell agonist® (*Bicycle* TICA®) molecules on the tumor immune microenvironment upon treatment of tumor bearing mice and the kinetics of tumor immune microenvironment modulation that ultimately leads to the robust anti-tumor activity in preclinical models.

We have used several different CD137 agonizing Bicycle® TICAs for this work. Based on the preclinical data we have gathered across our different CD137 agonizing Bicycle® TICAs, we believe that the mechanistic insights can be extended from one molecule to the next, barring the requirement of the appropriate tumor antigen expression for each one of the Bicycle® TICAs. Animal studies were performed according to the guidelines approved by the IACUC of WuXi AppTec (Beijing, China), following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care.

INTRODUCTION

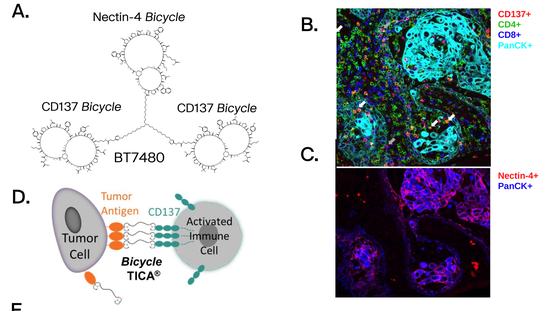


Figure 1: General concept of a CD137 *Bicycle*® TICAs using BT7480 as an example. Due to the synthetic nature of the *Bicycle* TICA™ molecules, both tumor antigen binding *Bicycles* and immune engager *Bicycles* can be easily mixed and matched with different linker lengths and stoichiometries of the *Bicycle* binders. (A) BT7480 is a fully synthetic, hetero-trimeric conjugate with one Nectin-4 and two CD137 binding *Bicycles*. CD137 is expressed by immune cells (B) and Nectin-4 is expressed on cancer cells (C) in a variety of solid tumor types[4-7], shown here in Head and Neck squamous cell carcinoma. (D) We hypothesized that by using Nectin-4 on cancer cells, BT7480 would be able to cluster and activate CD137 on immune cells in the tumor microenvironment. (E) We have used BT7480, BT7455 (EphA2 targeted *Bicycle* TICA®) and BCY12491 (another EphA2 targeted *Bicycle* TICA™) in these studies. Their anti-tumor activities were determined in MC38-Nectin-4 tumor model (BT7480) or MC38 tumor model (BY7455 and BCY12491) in huCD137-C57Bl/6 mice. Grayed area indicates the duration of treatment, CR denotes the number of complete responders.

Note: See our poster 1340 in this meeting to learn more about BT7455.

RESULTS

Treatment with CD137 *Bicycle*® TICAs led to infiltration of CD8+ cells into tumor tissue within 6 days of treatment initiation

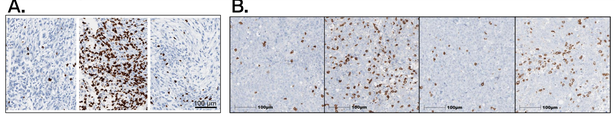


Figure 2: (A) Tumor harvested at 144-hours after treatment of MC38 bearing mice with vehicle, 15 mg/kg BCY12491 or NB-BCY (BCY13626) Q3D and stained for mouse CD8 are shown. (B) Tumor harvested at 144-hours after treatment of MC38-Nectin-4 bearing C57Bl/6 mice with vehicle, 5 mg/kg BT7480 or NB-BCY (BCY12797) at 0 and 24 hours or 2 mg/kg anti-CD137 antibody agonist Q3D and stained for mouse CD8 are shown.

Treatment with CD137 agonizing *Bicycle*® TICAs led to increases in cytotoxic cell scores and macrophage scores over time

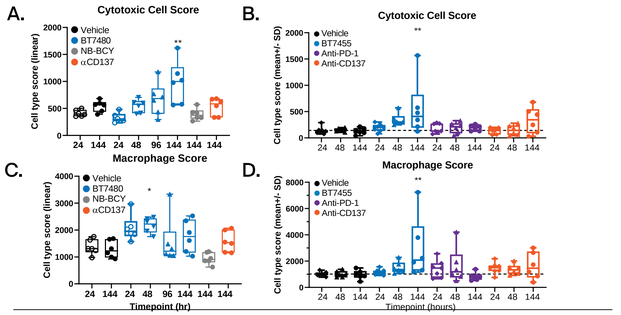


Figure 3: NanoString analysis of tumors show the effect of BT7480 and anti-CD137 antibody (Urelumab analogue) or BT7455, Anti-PD-1 RPM1-14 or Anti-CD137 on the cytotoxic cell (probe set: Ctsw, Gzma, Gzmb, Klrb1, Klrd1, Klrk1, Nkg7 and Prf1) (A and B) and macrophage (probe set: CD163, CD68, CD84 and Ms4a4a) (C and D) content. *p<0.05, **p<0.01, one-way analysis of variance with Dunnett's or Sidak's post-test.

Initial increases in cytotoxicity, apoptosis, and interferon signaling scores by CD137 *Bicycle* TICA™ are dependent on CD8 -cells

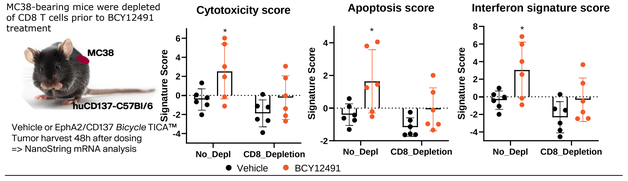
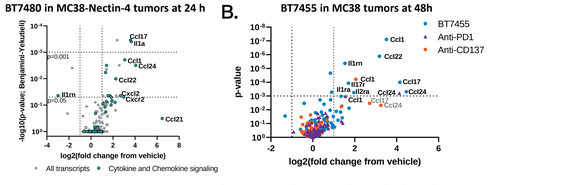


Figure 4: Cytotoxicity, apoptosis and interferon signaling scores were measured 48 hours after vehicle or 15mg/kg BCY12491 treatment in MC38 tumors from huCD137-C57Bl/6 mice or in huCD137-C57Bl/6 mice that had been depleted of CD8 –cells prior to treatment initiation. *p<0.05: 2way ANOVA with Sidak's post-test.

RESULTS

Similar increases in cytokine and chemokine signatures can be observed shortly (24-48h) after treatment with different CD137 *Bicycle*® TICAs in multiple mouse tumor models



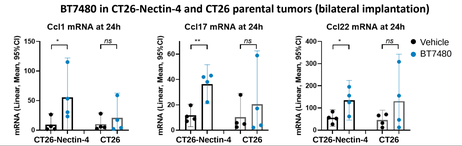


Figure 5: (A) MC38-Nectin-4 tumors were harvested from huCD137-C57Bl/6 mice 24h after vehicle or BT7480 (5 mg/kg) treatment and (B) MC38 tumors were harvested from huCD137-C57Bl/6 mice 48h after vehicle, BT7455 (8 mg/kg, 0 & 24h), Anti-PD1 RMP1-14 10 mg/kg or Anti-CD137 (Urelumab analogue) 2 mg/kg treatment. (C) CT26 and CT26-Nectin-4 tumors were harvested from huCD137-Balb/c mice 24h after vehicle or BT7480 (5 mg/kg) treatment. Transcriptional analysis was performed on tumor totRNA using NanoString mouse IO 360 panel. (A) Vast majority of transcriptional changes 24h after BT7480 treatment are observed in transcripts associated with cytokine and chemokine signaling. (B) More significant changes in cytokine and chemokine transcript are seen after BT7455 –treatment compared to Anti-PD-1 or Anti-CD137 –treatment.

Initial induction of Ccl1, Ccl17 and Ccl22 expression after CD137 *Bicycle* TICA™ treatment is not dependent on CD8+ cells

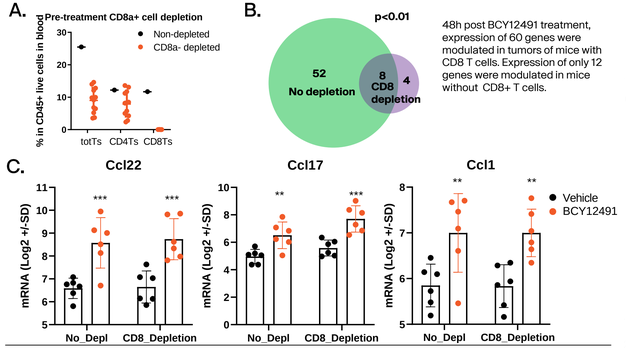


Figure 6: Increase in T cell chemotactic cytokines (Ccl1, Ccl17 and Ccl22) was not dependent on CD8+ T cells. (A) CD8 –cells were depleted from tumor bearing mice prior to 15 mg/kg BCY12491 treatment as shown by FACS from blood. Transcription of 4 genes (including Ccl1, Ccl17 and Ccl22) were uniquely modulated in CD8 –depleted mice. (C) Levels of Ccl22, Ccl17 and Ccl1 mRNA are shown from CD8 -depleted and non-depleted mice. **p<0.01, ***p<0.001: 2way ANOVA with Sidak's post-test.

RESULTS

BT7480 treatment leads to increase in Ccl17 and Ccl22 mRNA positive cells in MC38-Nectin-4 tumor tissue

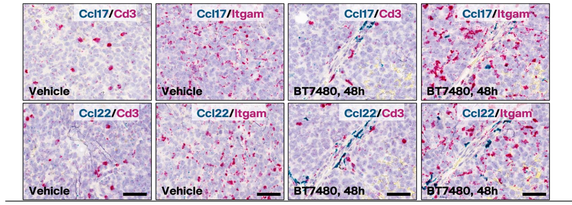


Figure 7: RNA-ISH images show Ccl17 and Ccl22 mRNA (in blue) positive cells in tissues harvested 48h after initiation of BT7480 treatment (5mg/kg at 0 and 24h) when compared to vehicle. No/little co-localization of Ccl17 or Ccl22 mRNA can be seen with Cd3 (red) or Itgam (red) mRNA.

Myeloid cells including monocytes, macrophages and dendritic cells can be the main sources of cytokines modulated by CD137 agonizing *Bicycle*® TICAs

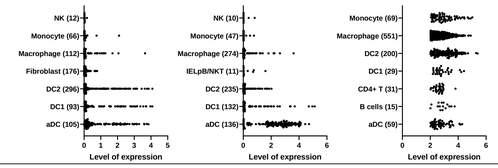


Figure 8: MC38 bearing huCD137-C57Bl/6 mice were treated with vehicle or 15 mg/kg BCY12491. Tumors were harvested 48 hours later for scRNAseq and a total of about 45K cells were sequenced. Markers for cell type identification for the preliminary analysis derive from modified (Park et al. Science 2021). Cell populations for top 1000 gene expressing cells from vehicle treated tumors are shown for three of the transcripts of interest. Preliminary analysis reveals that DC1 (XCR1+CLEC9A+), DC2 (SIRPA+CLEC10A+) and aDC (LAMP3+CCR7+) cell populations contribute significantly to Ccl17 and Ccl22 transcription. Ccl24 is also significantly expressed across monocytes and macrophages.

CONCLUSIONS

Altogether, these data highlight that CD137 *Bicycle*® TICAs like BT7480 and BT7455 may impact immune cell populations beyond T cells. We have observed an increase in several cytokines and chemokines in response to CD137 *Bicycle*® TICAs across several tumor models and demonstrated that some of these changes are independent of CD8+ cells. These findings with CD137 Bicycle® TICAs raise the interesting possibility, that abundant T cell infiltrates may not be required for *Bicycle*® CD137 agonists to initiate tumor rejection. This may broaden the patient population that may benefit from CD137 *Bicycle* TICA® treatment to also include myeloid-rich tumors with lower T cell infiltration ("colder" tumors).

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