A Therapeutic Strategy to Inhibit Wnt Signaling also Reprograms Breast Tumor-Immune Cell Interactions: Perspectives for Conferring Immune Checkpoint Inhibitor Susceptibility

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Abstract

Excessive Wnt signaling is associated with 1) poor prognosis in triple negative breast cancer (TNBC) and other cancers and 2) immune checkpoint inhibitor resistance, thus limiting its therapeutic application. From our previous work and Wnt signaling principles, we devised CHA1 as an inhibitor of Wnt signaling. CHA1 combines the green tea catechin EGCG (Epigallocatechin-3-gallate) and the DNA methyltransferase inhibitor Decitabine, which have been used in numerous clinical trials and FDA-approved for other cancers, respectively. Our investigations showed that CHA1 treatment (but not EGCG or Decitabine alone) reduced primary tumors and metastases in TNBC xenografts and decreased Wnt signaling. Unexpectedly, CHA1 treatment also re-programmed intrinsic tumor properties for antigen presentation and immune cell infiltration. The implications governing tumor-immune cell interactions are discussed in the context of increasing checkpoint inhibitor susceptibility.

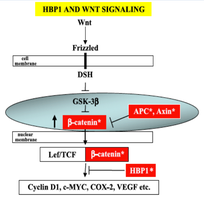
Both immune-compromised and immune-competent TNBC preclinical models were used for mechanistic elaboration. In CHA1-treated human TNBC tumors in immune-compromised mice, biochemical and RNA seq analyses showed that Wnt signaling was decreased due to induction of Wnt pathway inhibitors (e.g. SFRP1, DKK1, HBP1). The RNA seq analysis also revealed induction of >100 genes for antigen presentation and associated processes. MHC staining of CHA1-treated tumors verified the genotypic changes—which were also accompanied by robust gamma interferon signaling. All results were recapitulated in the immune-competent 4T1 syngeneic TNBC model. We also observed large increases in tumor-infiltrating CD8+ T cells with CHA1 treatment. Lastly, recent reports collated the molecularly disparate properties of a "cold-to-hot" transition, in which "hot" tumors have increased immune cell infiltration and sensitivity to checkpoint inhibitors. Remarkably, the TNBC tumors (typically "cold") exhibited the unrelated list of "hot" tumor properties after CHA1 treatment: 1) epigenetic reprogramming; 2) suppressed Wnt signaling; 3) E-cadherin and epithelial marker re-expression; 4) CD8+ T-cell enrichment; 5) increased tumor antigen presentation properties; and 6) increased tumor PDL-1 expression.

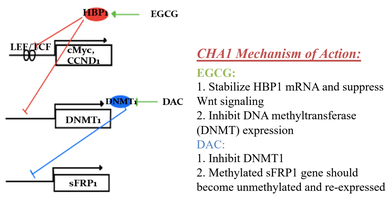
The CHA1 mechanism is consistent with a global reprogramming of intrinsic tumor properties triggered by differential Wnt and interferon signaling. CHA1 engages the fundamental processes that regulate tumor-immune cell dynamics, which, in turn, govern immune cell infiltration and determine checkpoint inhibitor sensitivity. Our work additionally establishes a molecular framework for assessing compounds that engage a fundamental "cold-to-hot" tumor reprogramming, which may predict new immune checkpoint inhibitor sensitivity. Thus, CHA1 treatment may reprogram tumor-immune cell dynamics to significantly expand the spectrum of TNBC and other tumors that can be efficaciously treated with immune checkpoint inhibitors (e.g. anti-PDL-1 and/or anti-PD1).

Background

The role of HBP1 in Wnt/β-catenin signaling

The Yee lab has identified HMG-box transcription factor 1 (HBP1) as a Wnt repressor and has shown that the expression of HBP was deleted, mutated or decreased in TNBC [1]. HBP1 inhibits Wnt signaling by binding to the LEF/TCF complex and prevents its function as a transcriptional activator [2]. In addition, the Yee lab has found that there was functional loss of HBP1 in clinical invasive breast cancer samples [1]. It also has been found that HBP1, by suppressing Wnt signaling, inhibits tumorigenesis of mammary gland cells [3]. The survival rate for patients with invasive breast cancer was reduced by 30% when the disease is accompanied by functional loss of HBP1 [3]. Hence, targeting HBP1 could be a potential candidate for anti-TNBC therapy.





CHA1 Mechanism of Action:

EGCG:
1. Stabilize HBP1 mRNA and suppress Wnt signaling
2. Inhibit DNA methyltransferase (DNMT) expression

DAC:
1. Inhibit DNMT1
2. Methylated sFRP1 gene should become unmethylated and re-expressed

Proposed CHA1 Outcomes:

1. Inhibition of Wnt signaling
2. Decreased tumor growth
3. Decreased tumor cell invasion and metastasis

TNBC Human Xenograft Model (Immune Compromised)

CHA1 Suppresses Tumor Growth

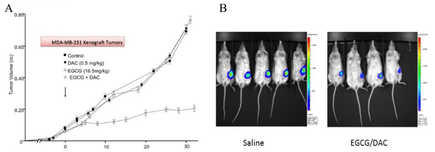


Figure 1. CHA1 Inhibits Tumor Growth. **A.** The LM1 subclone of MDA-MB-231 cells tagged with luciferase was implanted orthotopically into NOD-SCID mice. At least 5 mice were treated with CHA1 for the indicated time. **B.** Bioluminescence imaging of CHA1 treated xenograft.

TNBC Human Xenograft Model (Immune Compromised)

CHA1 Suppresses Metastases

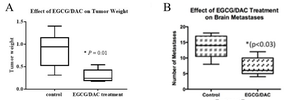


Figure 2. CHA1 Decreases Tumor Weight and Metastases. **A.** MDA-MB-231 cell tagged with luciferase was implanted orthotopically into NOD-SCID mice. At least 6 mice were treated with CHA1 and 5 mice treated with saline for the indicated time. Tumor weights were measured after 3 weeks of treatment. **B.** Orthotopic implants of the IS13 brain-metastasizing variant of MDA-MB-231 cells, tagged with GFP, were treated with CHA1 or saline for 2 weeks. GFP-labeled metastatic foci were counted on dissected brain.

CHA1 Inhibits Wnt Signaling

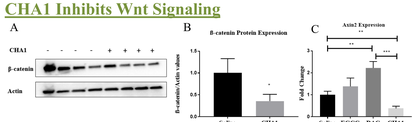


Figure 3. CHA1 Inhibits Wnt Signaling. **A.** β-catenin western blot of 4 untreated and 4 CHA1 treated tumors dissected from MDA-MB-231 human xenografts. **B.** Quantification graph combing data from three western blot each with 4 untreated and 4 treated samples. Actin was used as a loading control. **C.** Axin2 gene expression was tested by qRT-PCR after EGCG, DAC and CHA1 treatment of tumors from MDA-MB-231 human xenograft mice. * significant decrease in protein level after CHA1 treatment; P<0.01. ** significant decrease in fold change after CHA1 treatment; *P* < 0.005. *** significant decrease in fold change after CHA1 treatment ; *P* < 0.001. Data represented as mean ± SEM.

Bioinformatic Analysis: Human Tumors in an Immune Compromised Environment are Reprogramed by CHA1 Treatment to Become Antigen Presenting Cells

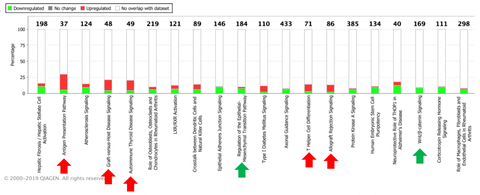


Figure 3. Bioinformatic Analysis of CHA1-Treated Tumors. RNA from tumors (Fig. 1) were subjected to RNA Seq and analyzed by IPA. The antigen presentation pathway is activated, indicating upregulation of genes involved in the anti-tumor immune response. Wnt signaling is downregulated, confirming the CHA1 mechanism. Epithelial adhesion junction (EMT process) is suppressed, which validates the decrease in cell motility. Green means down-regulated genes and red means up-regulated genes.

CHA1 Induces Antigen Presentation

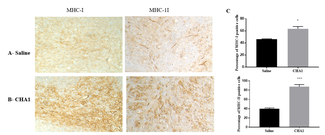
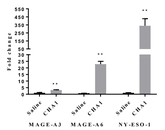


Figure 4. CHA1 Increases MHC-I and MHC-II. Sections of tumors were stained with pan MHC-I antibody or anti-MHC-II antibody. Low level expression was observed in (**A**) control group group (Average Allred score =4-5) while strong MHC-I and MHC-II expression was observed in (**B**) CHA1 treated group (Average Allred score = 8). **C.** Graph of the percentage of MHC-I and MHC-II staining in control tumors and CHA1 treated tumors. * significant increase after CHA1 treatment. P < 0.05. *** significant increase after CHA1 treatment. *P* < 0.001 Bar is mean ± SEM

CHA1 Increases Immunogenicity

Figure 6. CHA1 Increases Cancer Testis Antigen Expression. mRNA for MAGE-A3, MAGE-A6 and NY-ESO-1 was measured by qRT-PCR in tumors from mice treated with CHA1 or saline control. It was tested in 5 treated and 5 untreated tumors. ** *P* < 0.01. Data represents as mean ± SEM



CHA1 Increases Apoptosis

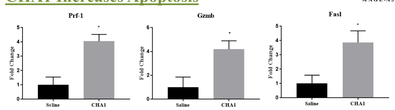


Figure 7. CHA1 Induces Apoptosis Genes in Human Xenograft. The gene expression of mouse Prf-1, Gzmb, and Fasl were tested by qRT-PCR after CHA1 treatment and saline treatment in human xenograft model. It was tested in 6 treated and 5 untreated tumors. * *P*< 0.005. Data represents as mean ± SEM.

TNBC Human Xenograft Model (CONTINUED)

CHA1 Activates Interferon Signaling

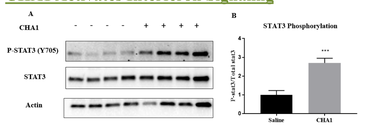


Figure 8. CHA1 Activates STAT3 Phosphorylation. **A.** Western blot analysis for P-STAT3 in tumors from 4 untreated and 4 CHA1 treated mice. **B.** Quantification from two blots. Actin was used as a loading control. *** significant increase in P-STAT3 level after CHA1 treatment. *P* < 0.005. Bars represent mean ± SEM.

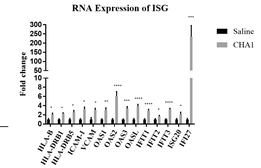
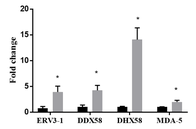


Figure 9. CHA1 Induces IFN Stimulated Genes (ISGs). The expression of each gene was measured by qRT-PCR after CHA1 and saline treatment. * *P*< 0.05, ** *P* < 0.01, *** *P* <0.005, **** *P* < 0.001. Bars represent mean ± SEM.

CHA1 Induces Viral Mimicry

Figure 10. CHA1 Induces Viral Mimicry Pathway Genes. The gene expression of ERV3-1 and dsRNA pattern recognition receptors DDX58, DHX58, and MDA5 was tested by qRT-PCR after CHA1 treatment and saline treatment. It was tested in 6 treated and 5 untreated tumors. * *P*< 0.05. Data represents mean ± SEM.



CHA1 Alters the Expression Profile of Immune Checkpoints

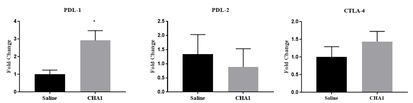


Figure 10. CHA1 Regulates Immune Checkpoints. The gene expression of PDL-1, PDL-2 and CTLA4 was measured by qRT-PCR after CHA1 treatment. It was tested in 6 treated and 5 untreated tumors. * *P*< 0.05. Data represents mean ± SEM.

TNBC Syngeneic Mouse Model (Immune Competent)

CHA1 Outcomes are Identical in Human and Mouse TNBC

1. *CHA1 Inhibits Wnt signaling*
2. *CHA1 Reduces Tumor Growth*
3. *CHA1 Induces JAK/STAT Signaling*
4. *CHA1 Induces INF Stimulated Genes*
5. *CHA1 Induces Antigen Presentation*
6. *CHA1 Viral Mimicry*

CHA1 Alters the Expression Profile of Immune Checkpoints

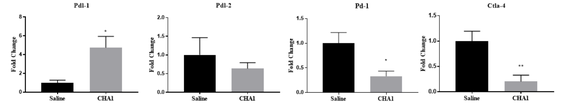


Figure 11. Immune Checkpoint Genes Altered by CHA1 Treatment. The gene expression of PDL-1, PDL-2, Pd1 and CTLA-4 was measured by qRT-PCR after CHA1 treatment. *P*< 0.05. ** *P*< 0.005. Data represents mean ± SEM.

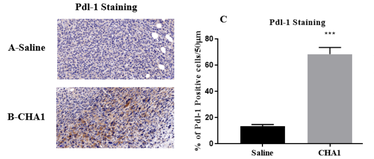


Figure 12. CHA1 Induces PDL-1 in 4T1 TNBC Tumors. Tumors were stained with anti-PDL-1 antibody. **A**. control group. **B.** CHA1 treated group. **C.** quantification graph of the percentage of PDL-1 positive cells in control and treated tumors. *** represents significant decrease after CHA1 treatment. *P* < 0.005. Data represents mean ± SEM

TNBC Syngeneic Model (CONTINUED)

CHA1 Induces Immune Cell Infiltration

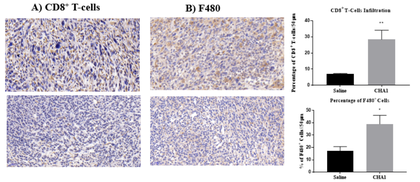
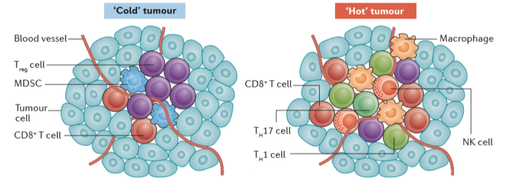


Figure 13. CHA1 Increases CD8+ T-cells and F480+ Cell Infiltration 4T1 TNBC Tumors. Treated and untreated tumors were stained with, **A**. anti-CD8+ antibody and **B.** anti-F480 antibody. **C.** Quantification graph of the percentage of CD8+ T-cells and F480+ cells in 5 control and treated tumors (mean ± SEM). * significant increase *P* < 0.05. ** significant increase *P* < 0.001.

CHA1 Re-programs Tumor Cells and May Confer Sensitivity to Immune Checkpoint Inhibitors

Converts cold tumors into hot tumors	Alters key signaling pathways and cellular processes	Reprograms a tumor to have antigen presentation properties and increased immune cell infiltration



Biological characteristics	• Epigenetic silencing • Active β-catenin signalling • Mesenchymal-like cells • Stem cell-like cells • Less-differentiated cells	✓• Epigenetic reprogramming ✓• Suppressed β-catenin signalling ✓• Epithelial cells ✓• Highly differentiated cells ✓• High PDL1 expression	Actions of CHA1
Immunological characteristics	• Enriched in immunosuppressive cytokines • High numbers of T_{reg} cells and MDSCs • Few T_H1 cells, NK cells and CD8+ T cells • Few functional APCs	• Enriched in T_H1-type chemokines ✓• High numbers of effector immune cells (T_H1 cells, NK cells and CD8+ T cells) ✓• High numbers of functional APCs	
	• Endogenous retroviruses silent • Dysfunctional JAK/STAT/IFN	✓• Induction of viral mimicry ✓• Active JAK/STAT/IFN	

Adapted from Nagarsheth N, Wicha MS, Zou W. Nat Rev Immunol. 2017 Sep;17(9):559-572.

CHA1 Functions in "Cold" to "Hot" Transitions

1. Inhibition of Wnt Signaling
2. Alteration of Epithelial-Mesenchymal Transitions
3. Re-expression of Endogenous Retrovirus Expression
4. Activation of Viral Mimicry Mechanisms
5. Activation of JAK/STAT Signaling
6. Activation of Interferon Stimulated Gene Signature (e.g. PDL-1, MHCs, etc)
7. Re-expression of selected Cancer Testis Antigens
8. Increased Tumor Immune Cell Infiltration (e.g. $CD8^+$ T-cells).

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