2880

INHIBITION OF HDAC6 AND HDAC11 HAS AN OPPOSITE EFFECT ON INFLAMMATION AND THE FUNCTIONAL PHENOTYPE OF MACROPHAGES IN THE TUMOR MICROENVIRONMENT

Manasa Suresh[1], Hawa Coulibaly[1], Xintang Li[1], David Quiceno-Torres[1], Nithya Gajendran[1], Karen Tan[1], Matias Hepp[2], Karthik Musunuri[2], Satish Noonepalle[1], Alejandro Villagra[1]

1. Georgetown University, Lombardi Comprehensive Cancer Center, Washington, DC. 2. Universidad Catolica de la Santisima Concepcion, Concepcion, Chile *3 Avstera Therapeutics, Pennsylvania, USA*

Abstract

Pan-histone deacetylase (HDAC) inhibitors have been used as anticancer agents due to their cytotoxicity over transformed cells. However, studies from our group have reported that intervention of class-specific HDAC inhibitors has anticancer properties by differentially affecting immune-related pathways in macrophages and influencing their pro- or anti-inflammatory phenotype and function. For example, specific HDAC6 inhibitors demonstrated promising antitumor effects by suppressing tumor-promoting M2 macrophages in the tumor microenvironment (TME). In contrast, inhibition of HDAC11 promoted the anti-inflammatory phenotype of macrophages. Besides the divergent roles and distinct signaling pathways, a comprehensive study on the effect of HDCA6 and HDAC11 inhibition on macrophage phenotype and the implications on TME and inflammation is warranted. In this study we characterize the effect of HDAC6 and HDAC11 inhibition on the phenotype and function of macrophages. Bone marrow-derived macrophages (BMDMs) isolated from WT, HDAC6KO, and HDAC11KO C57BL/6 mice were pre-treated with HDAC6 (Nexturastat A) or HDAC11 (FT895) inhibitors prior to polarizing them to M1 and M2 phenotypes. The changes in gene expression were identified by RNA-seq analysis. Furthermore, single-cell secretome analysis using the Isoplexis platform was performed for BMDMs on a panel of cytokines and chemokines. Transcriptomic analysis revealed that HDAC6 inhibition in M1 polarized BMDMs sustained inflammatory gene signature while suppressing the tumor-promoting and anti-inflammatory genes in M2 macrophages. This supported our previous reports where HDAC6i increased M1/M2 ratio in murine melanoma models. In contrast, HDAC11 inhibition in M2 macrophages upregulated classical M2 markers, whereas M1-associated gene signature was minimally affected. These results were recapitulated in HDAC6KO and HDAC11KO macrophages, suggesting the high specificity of the inhibitors. Further validation at protein level by single-cell secretome analysis indicated that HDAC11 inhibition in M2 polarized BMDMs increased secretion of growth factors such as Egf and Pdgf and anti-inflammatory cytokines such as Il10 and Il13. On the contrary, HDAC6 inhibition upregulated inflammatory cytokines such as Ifn-γ and Tnf-α and T-cell recruiting chemokine Cxcl10 in the M1 phenotype.

Introduction and Relevance

Histone deacetylases (HDACs) have been recognized to have implications for cancer progression, and several pan-HDAC inhibitors have been used in the clinical setting as a treatment option based on their cytotoxic effects . However, the modest efficacy of such broad inhibitors and the treatment-associated toxic side effects have hindered their use in combination immunotherapies against different malignancies. We have previously demonstrated that class-specific HDAC inhibitors (HDACis) can overcome these drawbacks. Furthermore, ultra-specific HDACis, such as HDAC6is, can modulate the function of macrophages towards antitumor properties.

An oversimplified classification of macrophages includes proinflammatory M1 and anti-inflammatory M2 phenotype. Tumor-associated macrophages (TAMs), with their predisposition towards M2 function, play an essential role in shaping antitumor immunity and the high infiltration of TAMs is usually associated with poor prognosis in several types of cancer. Therefore, the M1/M2 macrophage ratio has become an emerging determinant of antitumor immunity. Due to the plastic nature of TAM phenotypes, strategies to alter TAMs into antitumor M1 phenotype or diminish pro-tumor M2 phenotype within the tumor microenvironment (TME) have gained prominence.

Here we show that inhibition of two HDACs, HDAC6 and HDAC11, using ultra-specific inhibitors has an opposite effect on macrophage phenotype and function. These observations emphasize the significance and benefit of using class-specific HDACis over pan-HDACis.

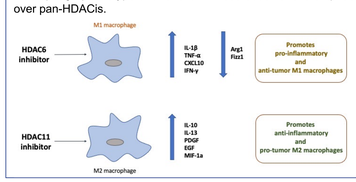

Fig. 1 | Opposite effects of HDAC6 and HDAC11 inhibition on macrophage phenotype and function. HDAC6 inhibition increases the proinflammatory M1 phenotype of macrophages and promotes antitumor function. HDAC11 inhibition increases anti-inflammatory cytokines and growth factors that promote the M2 phenotype and the protumor function of macrophages.

HDAC6 inhibition modulates macrophage phenotype *in vitro*

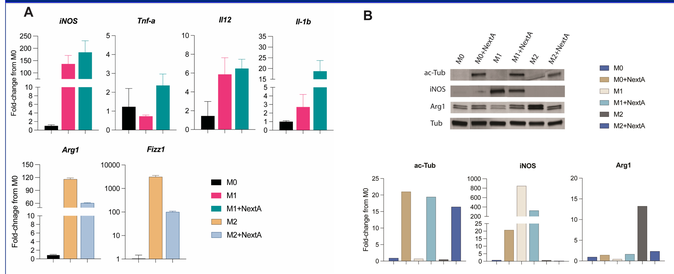

Fig. 2 | HDAC6 inhibition reduces M2 phenotype of bone marrow-derived macrophages. BMDMs treated with HDAC6i, Nexturastat A (NextA) were analyzed by **A.** qRT-PCR for quantification of key genes associated with M1 (*iNOS, Tnf-α, Il-12,* and *Il-1b*) and M2 (*Arg1*, *Fizz1*, and *TGF-β*) phenotype of macrophages. **B.** western blot for acetylation of tubulin and changes in levels of Arg1 and iNOS.

HDAC11 inhibition increases anti-inflammatory phenotype of M2 macrophages *in vitro*

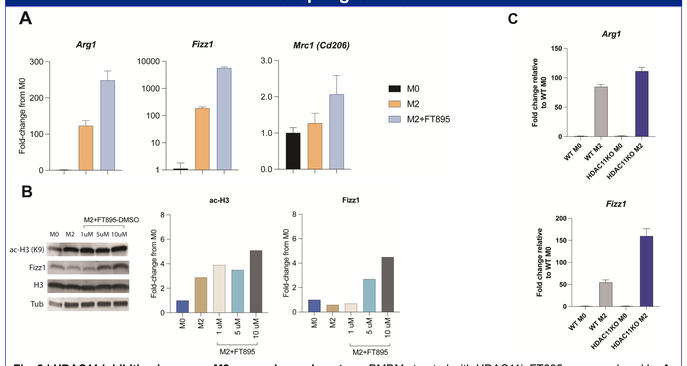

Fig. 3 | HDAC11 inhibition increases M2 macrophage phenotype. BMDMs treated with HDAC11i, FT895, were analyzed by **A.** qRT-PCR for quantification of key genes associated with M2 (*Arg1*, *Fizz1*, and *Mrc1*) phenotype of macrophages. **B.** western blot for acetylation of histone 3 and changes in levels of Fizz1; **C.** The expression of M2 markers, *Arg1* and *Fizz1*, were quantified in BMDMs isolated from wildtype (WT) and HDAC11 knockout (HDAC11KO) mice.

Transcriptome analysis reveals that HDCA6 and HDAC11 inhibition have opposite effects on M2 macrophage markers.

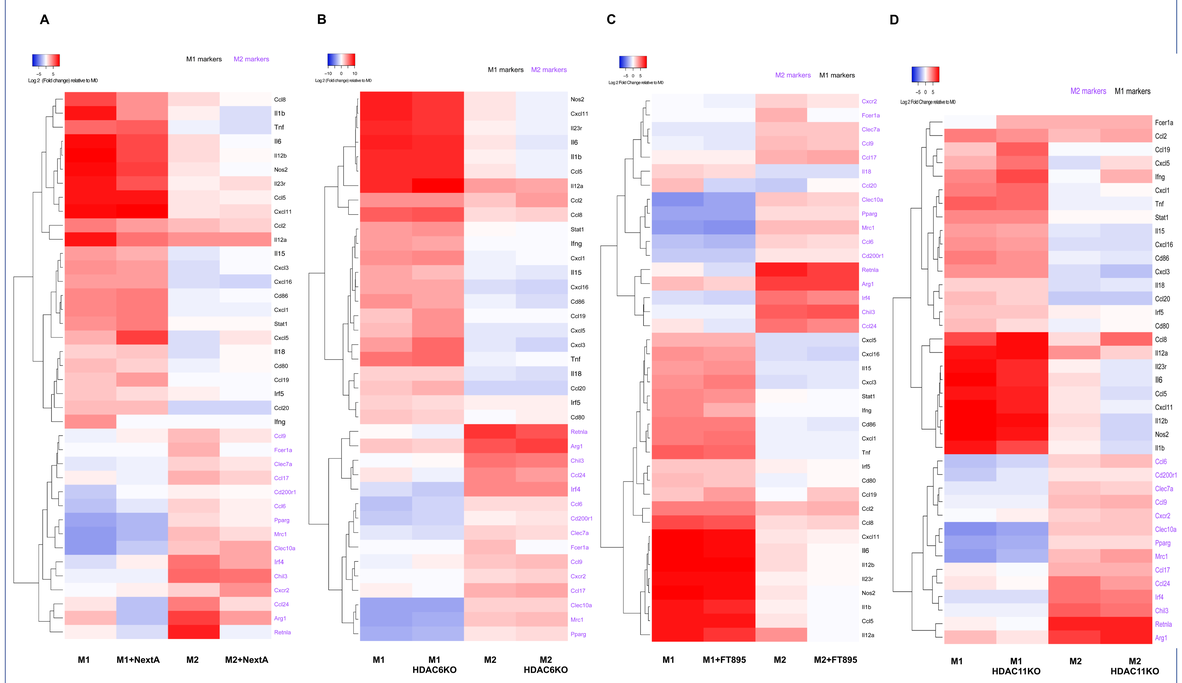

Fig. 4 | Transcriptome analysis of bone marrow-derived macrophages for M1 and M2 macrophage markers changes after HDAC6 and HDAC11 inhibition. Bone marrow-derived macrophages from wildtype and HDCA6 or HDAC11 knockout C57BL/6 mice were polarized to M1 and M2 phenotype with or without treatment with HDAC6i (NextA) and HDAC11i (FT895) and applied to RNA sequencing. The heat map represents the changes in M1 and M2 macrophage gene signature expression associated with HDAC6 inhibition by **A.** NextA and **B.** HDAC6KO; and with HDAC11 inhibition by **C.** FT895 and **D.** HDAC11KO. The low to high expression of genes is indicated by a range of blue to red color.

Single-cell secretome analysis indicates distinctive effects of HDAC6 and HDAC11 inhibition on growth factors, cytokines, and chemokines.

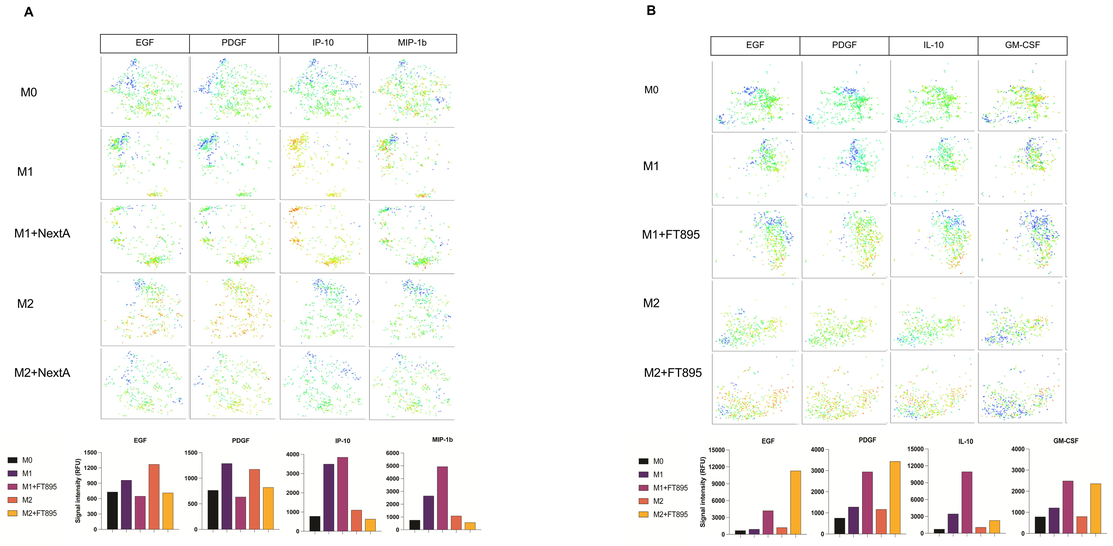

Fig. 5 | Single-cell secretome analysis. Bone marrow-derived macrophages from wildtype C57BL/6 mice were treated with HDAC6 and HDAC11 inhibitors, NextA and FT895, respectively, and polarized to M1 and M2 phenotypes. The cells were applied to single-cell secretome analysis using the Isoplexis platform. The 2D T-SNE plot and the corresponding graphs show the changes in secreted growth factors (EGF and PDGF), cytokines (IL-10), and chemokines (IP-10 and MIP-1b) associated with **A.** Next A and **B.** FT895 treatment.

Targeted application of class-specific HDAC6 inhibitor *in vivo*

HDAC6 inhibitor pre-treated macrophages for cell therapy: For evaluating the direct effect of HDAC6 inhibition on TME, BMDMs polarized to M1 phenotype with or without NextA pre-treatment were administered to SM1 tumors. When compared to the vehicle control, animals that received M1 macrophages pre-treated with NextA showed a significant reduction in tumor volume and improved survival rate. In support of this method of adoptive cell therapy, the endpoint tumors from the NextA pre-treated M1 macrophages had a higher M1/M2 ratio than other groups. Therefore, HDAC6 inhibition prevented the switch from M1 to M2 phenotype of macrophages in the TME.

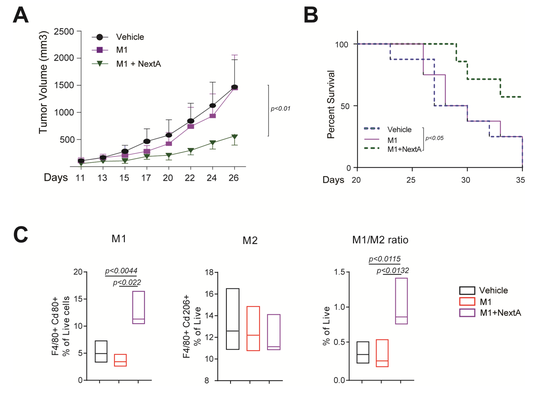

Fig. 6 | Adoptive cell therapy with macrophages pre-treated with HDAC6 inhibitor. Intra-tumoral injection of NextA treated M1 macrophages showed a significant reduction of SM1 tumor volume **(A)** when compared to vehicle and M1 polarized macrophages. The Kaplan-Meier survival curve **(B)** indicates improved survival of mice that received M1 macrophages treated with NextA. Additionally, increased M1/M2 ratio was observed in flow cytometry analysis **(C)** for in macrophages isolated from endpoint tumors of mice that received M1+NextA treatment.

Materials and Methods

Isolation and maintenance of BMDMs: BMDMs were isolated from C57BL/6 wildtype, HDAC6KO, and HDAC11KO mice and maintained in complete RPMI media containing 20 ng/mL of mouse-MCSF for 4 days and for additional 3 days in media without mouse-MCSF.

Treatment and polarization of BMDMs: BMDMs were treated overnight with inhibitors of HDAC6 (NextA), and HDAC11 (FT895), and again two hours before polarization. LPS and IFN-γ were used for M1 macrophage polarization, and IL-4 and IL-13 cytokines were used for M2 polarization.

qRT-PCR and western blot analysis: Cells were harvested between 12-16 hours after polarization for RNA isolation and between 22-24 hours for protein isolation. cDNA prepared from RNA was applied in qRT-PCR assay using the BioRad. Proteins, including tubulin, acetyl tubulin, iNOS, Arginase 1, Fizz 1, histone 3, and acetyl-histone 3 were quantified by western blot.

RNA sequencing and single-cell secretome: The expression of genes obtained from RNA sequencing data was quantified using a software called SALMON and the differential expression was analyzed by edgeR tool. Single-cell secretome analysis was performed using the Isoplexis platform with the mouse innate immune panel.

Adoptive cell therapy: BMDMs isolated from wildtype C57BL/6 mice were treated with 5 uM of NextA and polarized to M1 phenotype using LPS and IFN-g. 24 hours later, cells were scrapped and administered as intra-tumoral injection to SM1 tumor-bearing C57BL/6 wildtype mice.. The end point tumor was obtained approximately 2 weeks from the initial engraftment of SM1 cells. Macrophages were isolated from tumors for flow cytometry analysis.

Conclusion/Summary

- *In vitro* treatment of BMDMs with HDAC6 inhibitor, NextA, increases the proinflammatory, M1 phenotype of macrophages and more significantly reduces the pro-tumor M2 phenotype of macrophages.
- On the contrary, HDAC11 inhibition with FT895 enhances the anti-inflammatory M2 phenotype of macrophages.
- The RNA sequencing and single-cell secretome analyses indicate that HDAC6 and HDAC11 affect macrophage function in diametrically opposite directions.
- These results underscore the importance of using class-selective inhibitors of HDACs over pan-HDAC inhibitors and the potential of ultra-specific HDAC6 inhibitor as a therapeutic option to control macrophage phenotype in cancer and other conditions.

Future directions

- Based on our observations regarding class-specific HDAC inhibitors, we have expanded our work in adoptive cell therapy using HDAC6 inhibitors. See AACR published abstract #900 for additional results from adoptive cell therapy.
- Encouraged by the results from macrophage-based ACT in murine melanoma SM1 model models, we plan to laterally expand this therapy to other cancer models including breast, lung, and prostate cancer.
- We have performed preliminary studies combining HDAC6 inhibitors and immune checkpoint inhibitors in the SM1 murine melanoma model.
- We plan to explore the function of HDAC11 inhibitors against chronic inflammation based on their ability to modulate macrophages towards M2 or anti-inflammatory phenotype.

Funding

This work has been supported by:
- NIH R01 R01CA249248
- Melanoma Research Foundation Team Award NSF1830941
- Cancer Research Institute 228514
- McCormick Foundation
- NIH R41CA217294