Abstract 4021

The class I HDAC inhibitor, mocetinostat, induces expression of PD-L1 and tumor antigen presentation machinery and modifies tumor immune cellular subsets providing a rationale for immune checkpoint inhibitor combinations

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Abstract

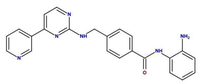
Immunotherapy has led to major treatment breakthroughs for a number of cancers including non-small cell lung cancer (NSCLC). Although initial responses to immune checkpoint inhibitors are promising, a significant percentage of patients do not respond or rapidly acquire resistance. Although the mechanisms underlying intrinsic and acquired resistance remain largely unexplained; the expression of programmed cell death-ligand 1 (PD-L1), lack of tumor cell capacity to effectively present neoantigens, and presence of immunosuppressive cellular subsets have been implicated as potential mechanisms. Histone deacetylase (HDAC) inhibitors have emerged as a class of agents that may combat checkpoint inhibitor resistance by reversing immune evasion and eliciting an anti-tumor activity through a multi-faceted immuno-stimulatory mechanism of action. Mocetinostat is a spectrum-selective Class I/IV HDAC inhibitor specifically targeting HDAC-1, -2, -3 and -11. The present studies were designed to explore mocetinostat's effect as an immune-enhancer and ultimately, to evaluate its potential to be used in combination with immune checkpoint inhibitors (e.g., PD-1/PD-L1 antagonists). Specifically, we assessed mocetinostat's effect on the expression of various immunomodulatory factors by tumor cells as well as its effect on immune cell sub-populations in the tumor microenvironment *in vivo*. Mocetinostat elicited a concentration-dependent increase in PD-L1 mRNA expression which translated into increased PD-L1 surface protein expression in a panel of NSCLC cell lines. In addition, mocetinostat elicited a concentration-dependent increase in expression of MHC-class I related polypeptide-related sequence A (MIC-A) and MIC-B, and cluster of differentiation 86 (CD86). Furthermore, mocetinostat induced expression of several human leukocyte antigen (HLA) gene complex family members including HLA-A, -B, -DRA, and -DPA among others. To determine the effect of mocetinostat on systemic and tumor immune cell subpopulations we treated CT26 tumor-bearing mice. Mocetinostat increased splenic CD4-positive T effector cells and tumor mature cytolytic CD8-postive T cells and at the same time decreased tumor FoxP3-positive T regulatory cells and CD11b/Gr1-positive myeloid-derived suppressor cells (MDSC). These data provide evidence that mocetinostat modulates key immune regulators both in tumor cells as well as in relevant immune cell types in the tumor microenvironment and provides strong rationale for combination with immune checkpoint inhibitors.

Background

Accumulating evidence indicates that tumors evade immune responses by down regulation of MHC molecules and tumor antigens or active suppression of anti-tumor immune responses at the site of the tumor by creating an immune suppressive tumor microenvironment. In this study, we sought to explore the anti-tumor activity of the combination of mocetinostat and an inhibitory PD-L1 antibody based on the following rationale:

- Tumors that lack expression of PD-L1 have a decreased likelihood of response to checkpoint inhibitors[1-3].
- Tumor cells down regulate antigen presentation machinery, including MHC class I molecules[4,5].
- HDAC inhibitors (HDACis) upregulate MHC class I and class II molecules, co-stimulatory molecules such as CD80, CD86 and CD40[6,7] and NKG2D ligands MICA and MICB[8]. MICA and MICB are Natural Killer (NK) cell stress receptors ligands and stimulate the activity of NK cells[9].
- Class I HDACis decrease immuno-suppressive Tregs and MDSCs whereas Class II HDACis have been shown to enhance Treg number and function[10-13]. Therefore, class II or pan-HDACis may have an immuno-suppressive effect in the TME while class I HDACis may stimulate the immune system by reducing these immuno-suppressive cell populations.
- HDACis stimulate immunologic tumor cell death[14].
- HDACis enhance the function of the cytotoxic CD8+ T cell subset[15,16].

Mocetinostat



Class I HDAC inhibitor of the benzamide class

- Anti-proliferative, pro-apoptotic HDAC 1, 2, 3 and 11 inhibitor
  - Inhibition of proliferation in a variety of solid tumors and hematopoetic malignancies and patients' tumor explants
  - Cell cycle arrest: G2/M arrest and sub-G1 accumulation, induction of apoptosis, dose-dependent↑$p21^{waf1/Cip1}$ in cancer cell lines
  - Dose-dependent induction of H3 and H4 acetylation in multiple cancer cell lines in vitro
- Differentiated from other HDAC inhibitors
  - Class I HDACis decrease Tregs and MDSCs that function as immune suppressors and induce tolerance to tumor antigens. Importantly, whereas class I HDAC inhibitors decreased the number and function of Tregs, pan-HDAC inhibitors enhanced Treg number and function
  - Demostrates a durable effect on histone acetylation lasting at least 72 hours post administration. This durable effect on acetylation was not observed with hydroxamate-based HDAC inhibitors in comparative nonclinical studies
- Clinical Summary
  - Evaluated on over 10 clinical trials and in over 400 patients resulting in a good understanding of the PK and safety profiles
  - Demonstrated signs of clinical activity and confirmed partial responses including both Hodgkin's' and non-Hodgkin's lymphoma, acute myeloid leukemia, and myelodysplastic syndrome patients as a single agent and/or in combination with 5-azacitidine
  - A clinical trial combining mocetinostat with the PD-L1 inhibitor durvalumab is planned to start enrolling NSCLC patients in Q2 2016

Methods

Gene expression analyses

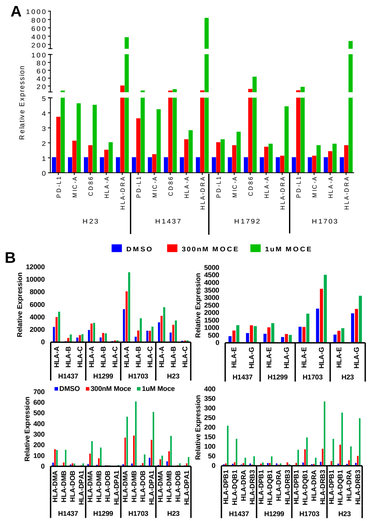
- NSCLC cell lines and CT26 tumor bearing mice were treated with mocetinostat. Cells were seeded in 10 cm dishes and incubated with various doses of mocetinostat or DMSO for 48 hours. RNA was extracted with the Qiagen RNeasy plus kit. 100 ngs were used with the Nanostring nCounter® PanCancer Immune Profiling Panel.
- Relative gene expression was determined for additional genes by quantitative reverse transcription PCR (qRT-PCR) in NSCLC cell lines after 48 hrs mocetinostat treatment. Data were normalized to actin, GAPDH and GUS-b and then normalized to DMSO treated samples.

In vivo studies

- The effect of mocetinostat on immune cell populations was determined using FACS analysis following treatment in the CT26 syngeneic colon cancer tumor model grown in Balb/c female mice. Mice were treated p.o. with vehicle (3) or 100 mg/kg mocetinostat (5) for 3, 6 and 9 days. Initiation of dosing was staggered to control for tumor size at take-down. Tumors were dissociated in a gentleMACS C tube and spleens were mechanically dissociated with syringe plunger. Stained samples were loaded into Attune Autosampler for FACS analysis. Flow cytometry markers included CD4+ and CD8+ T-Cells, FoxP3+ Regulatory T-Cells (Tregs), CD8+/Ki67+ and MDSCs (CD11b/Gr1+). Error bars represent standard deviation.
- In vivo efficacy of mocetinostat in combination with anti-mPD-L1 antibody was performed in the CT26 model. Thirteen mice per group were treated with an isotype control (LTF-2) Q2Dx5, anti-mPD-L1 (Clone 10F.9G2) antibody 10 mg/kg Q2Dx5, 100 mg/kg mocetinostat QDx14 and LTF-2 Q2Dx5, and 100 mg/kg mocetinostat QDx14 and anti-mPD-L1 antibody 10 mg/kg Q2Dx5 with mocetinostat treatment initiated 3 days prior to anti-mPD-L1. Tumor burden (mm3) was estimated from caliper measurements by the formula: Tumor burden (mm3) = (L x W2)/2, where L and W are the respective orthogonal tumor length and width measurements (mm).

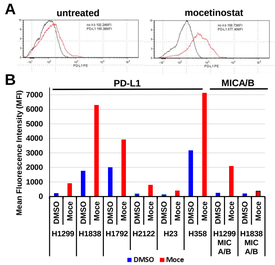
Effects of mocetinostat on evasion of immune surveillance in tumor cells

Figure 1. Mocetinostat upregulates expression of PD-L1, immune co-stimulatory molecules and antigen presentation machinery in NSCLC cell lines



A. NSCLC cell lines were treated with DMSO, 300 nM or 1 uM mocetinostat for 48 hrs *in vitro*. RNA was harvested and reverse transcribed. qRT-PCR was performed on the genes shown and normalized to housekeeping genes and DMSO control. **B.** NSCLC cell lines were treated as in A. 100 ngs RNA were ran on the nanoString™ PanCancer Immune Profiling Panel. Relative expression is shown and data were normalized using a panel of housekeeping genes.

Figure 2. Mocetinostat increases surface protein PD-L1, immune stimulatory molecules and antigen presentation machinery



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H1299	HLA-A,B,C	MIC-A,B	CD86
Vehicle	5574	271	114
300nm	6985	521	112
1um	6557	852	123

NCI-H1437	HLA-A,B,C	MIC-A,B	CD86
Vehicle	1749	229	119
300nm	1638	170	135
1um	1619	297	185

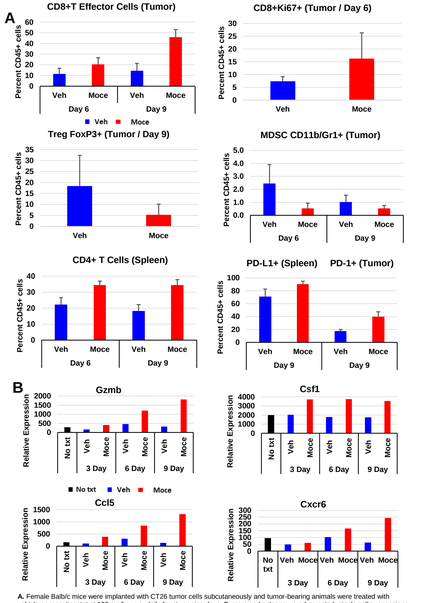
NCI-H1792	HLA-A,B,C	MIC-A,B	CD86
Vehicle	10874	326	106
300nm	13300	295	106
1um	13087	388	127

NCI-H2122	HLA-A,B,C	MIC-A,B	CD86
Vehicle	9440	123	128
300nm	9905	131	126
1um	8426	180	158

A. H2122 cells (NSCLC) were untreated or treated with mocetinostat 1 uM for 48 hrs then fixed and stained for surface PD-L1 protein expression and analyzed using FACS. The black line represents the no PD-L1 primary antibody control; the red line represents PD-L1 surface protein expression. A clear shift is observed with mocetinostat treatment. **B.** Mean fluorescence intensity (MFI) surface protein expression of PD-L1 and MICA/B across a panel of NSCLC cell lines following 48 hr treatment with DMSO control or 1 uM mocetinostat. **C.** MFI of additional immune signaling molecules treated as in **B**.

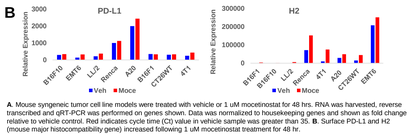
Effects of mocetinostat on key immune cell populations *in vivo*

Figure 3. Mocetinostat increases CD4+ and CD8+ cells and the expression of circulating factors and decreases immunosuppressive cell populations *in vivo*



A. Female Balb/c mice were implanted with CT26 tumor cells subcutaneously and tumor-bearing animals were treated with vehicle or mocetinostat at 100 mg/kg, p.o. daily for six or nine days. Tumors and spleens were harvested, single cell suspensions were prepared and immune cell populations were analyzed by FACS. Data shown as percent CD45 (pan haematopoetic marker) cells. Veh: vehicle; Moce: mocetinostat; Data shown represents average + standard deviation **B**. Inflammatory immune cytokine signaling components and secreted factors are regulated by mocetinostat. Tumor-bearing animals were treated as in A for three, six or nine days. RNA was purified from tumors and 100 ngs were analyzed by nanoString. Data were normalized to a panel of housekeeping genes. No txt: no treatment; Veh: vehicle; moce: mocetinostat.

Figure 4. PD-L1, pro-inflammatory molecules and antigen presentation machinery surface protein levels are upregulated by mocetinostat across a panel of mouse syngeneic models

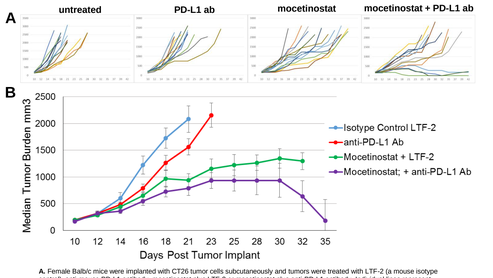


Mouse Model	Anti-Tumor Immunity						MHC				Complement		Peptide Processing	
	CD274	GZMa	IFNg	PRF-1	NOS2	H2-Aa	H2-K1	H2-DMa	H2-Q1	H2-T23	C3	CD74	TAPBP	TAP2
4T1	1	3	4	28	52	176	2	2	3	2	40	3	2	1
A20	10	1	1	11	1	6	5	6	4	2	3	2	1	1
B16F10	4	1	1	1	3	71	9	14	2	29	14	19	6	46
EMT6	1	2	1	3	3	4	N/A	1	20	1	0	2	5	1	0
LL/2	6	4	11	4	3	615	2	21	6	N/A	73	87	1	1
Renca	1	1	11	69	1	13	2	5	12	4577	1	4	1	1
B16F1	1	1	1	4	6	N/A	1	2	5	11	2	3	2	6
CT26WT	2	6	149	2	24	1376	7	16	18	0	546	52	3	1

A. Mouse syngeneic tumor cell line models were treated with vehicle or 1 uM mocetinostat for 48 hrs. RNA was harvested, reverse transcribed and qRT-PCR was performed on genes shown. Data was normalized to housekeeping genes and shown as fold change relative to vehicle control. Red indicates cycle time (Ct) value in vehicle sample was greater than 35. **B**. Surface PD-L1 and H2 (mouse major histocompatibility gene) increased following 1 uM mocetinostat treatment for 48 hr.

Anti-tumor effect of mocetinostat in combination with a PD-L1 checkpoint inhibitor

Figure 5. Mocetinostat + anti-PD-L1 antibody combination elicits significant anti-tumor activity in a syngeneic mouse tumor model



A. Female Balb/c mice were implanted with CT26 tumor cells subcutaneously and tumors were treated with LTF-2 (a mouse isotype control), anti-mouse PD-L1 antibody, mocetinostat plus LTF-2 or mocetinostat plus anti-PD-L1 antibody. Individual lines represent individual tumor volumes (y-axis = mm^3) over time (x-axis = days post tumor implant). **B.** Median tumor volumes of animals remaining in the cohort +/- sem.

Conclusions

- Mocetinostat upregulates PD-L1, co-stimulatory molecules, Class I and Class II MHC genes and the NKG2D receptor ligands MICA and MICB mRNA across several NSCLC cell lines tested. FACS analysis confirmed this translated into increased surface protein expression. Similar observations were made in mouse syngeneic model cell lines in vitro.
- Mocetinost increased CD8+ and CD8+/Ki67+ cells and decreased immunosuppressive Tregs and MDSCs in the tumor. CD4+ cells were also increased in the spleen following mocetinostat treatment.
- The combination of mocetinostat and an anti-mouse PD-L1 antibody resulted in greater antitumor activity compared to either agent alone.

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