Selective USP7 inhibitor enhances effector cytokines and killing capacity of T lymphocytes resulting in inhibition of tumor growth in colorectal cancer model

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Background

Ubiquitination, the addition of ubiquitin to a substrate, is a post translational modification critical to cell homeostasis. Expression of deubiquitinases (DUBs) can be abnormal in tumors and the tumor microenvironment, presenting DUBs as a potential important group of targets for anticancer therapeutic agents. High expression of USP7 is seen to be aberrant in several tumor indications promoting oncogenesis. Upregulation of USP7 predicts an unfavorable prognosis for various cancers, suggesting that USP7 inhibitors may be promising therapeutics for cancer patients.

USP7 regulates the levels of multiple proteins involved in cell cycle and immune response, particularly in the homeostasis of p53, a tumor suppressor protein. We have discovered a novel and selective USP7 inhibitor (USP7i) with nanomolar activity (IC50< 30nM) that acts through stimulating the immune response against cancer cells. Using primary macrophages and T cells we showed immunomodulatory properities of our USP7 inhibitor and its capability to enhance anti-tumor immunity and reduce TAM-associated markers, namely CCL2 and Arginase 1 (Arg1). We also utilized CT26 syngeneic murine model of aggressive colon carcinoma to demonstrate enhanced killing capacity and effector functions of immune cells after treatment of mice with USP7i.

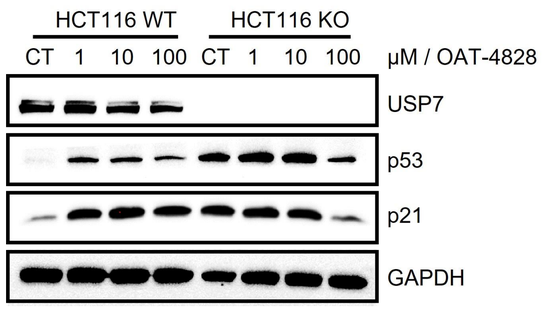
Methods

OAT-4828 is a selective USP7 inhibitor developed in Molecure with characterized pharmacokinetics and safety profile. HCT116 and CT26 cell lines were cultured in a recommended conditions by ATCC. BMDMs were differentiated for 6 days with M-CSF and then polarized to M1 with LPS+IFN-γ and to M2 with IL-4 for 24h. Naive CD4 T cells were isolated from spleens of naive mice using negative selection beads (Stem Cell) and activated by αCD3/28 and cultured in complete RPMI for 3 days. Levels of indicated cytokines were determined by ELISA (R&D) and gene expression was evaluated with Taqman system (ABI).

To evaluate efficacy of OAT-4828 in syngeneic colorectal cancer model CT26 cells were inoculated in the right flank of BALB/c mice. Tumor growth was measured every second or third day. OAT-4828 was administered twice daily by oral gavage starting from day 2 postinoculation. Human endpoint was estimated at 2000mm^3 tumor size. To determine killing capacity splenocytes from CT26-bearing mice were co-cultured with fluorescently-labelled CT26 cells in different ratios for 36h. Cell death was measured by flow cytometry. Efficacy of OAT-4828 was also tested in combination with αPD-1 treatment. Immunoprofiling of splenic cells and tumor infiltrating cells were performed by flow cytometry (Beckman Coulter).

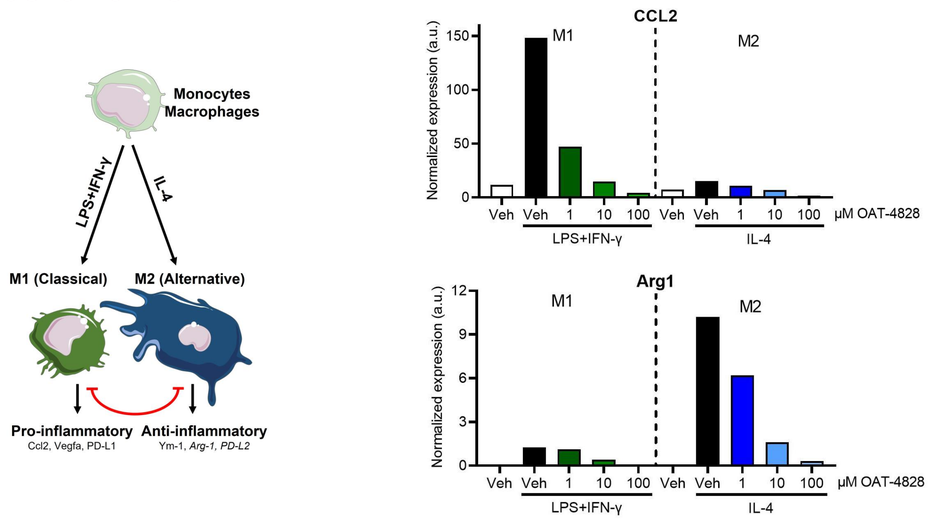
Results

Figure 1. Inhibition of USP7 leads to stabilization of p53 and upregulation of p21



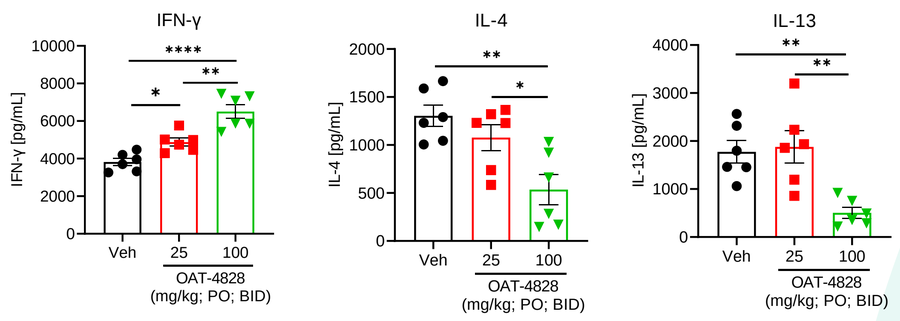
HCT116 WT cells were treated with increasing doses of USP7 inhibitor, OAT-4828, for 24h. USP7 KO HCT116 cells were used as a positive controls for activation of p53 and p21 signaling pathway. OAT-4828 treatment stabilized p53 protein and upregulated p21 expression.

Figure 2. OAT-4828 treatment reduces TAM-associated markers in a dose-dependent manner



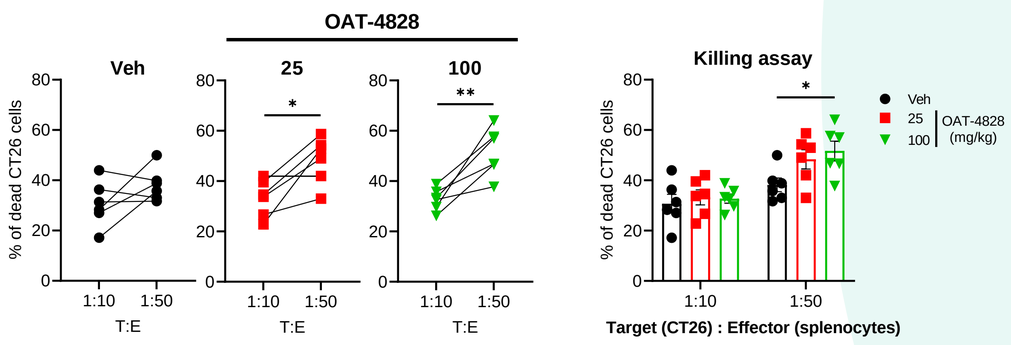
Bone marrow-derived macrophages (BMDMs) were polarized to M1- and M2-like macrophages and treated with increasing doses of USP7 inhibitor for 24h. Markers that are also associated with monocytes recruitment (CCL2) and immunosupression (Arg1) of a tumor microenvironment (TME) were evaluated by qRT-PCR. We showed that macrophages treated with OAT-4828 reduced levels of CCL2 and Arg1 from M1 and M2 cells in a dose-dependent manner.

Figure 3. Inhibition of USP7 promotes secretion of Th1- and reduces Th2-associated cytokines



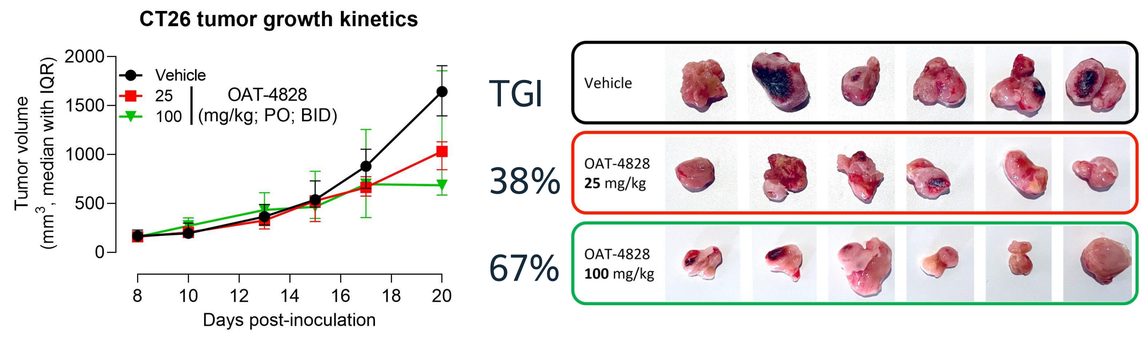
Isolated splenic CD4 T cells were stimulated with αCD3/28 and treated with different concentrations of OAT-4828 for 72h. Next, Th1- and Th2-associated cytokines were evaluated in the supernatants by ELISA. We demonstrated that inhibition of USP7 enhanced secretion of IFN-γ and reduced IL-4 and IL-13 from activated CD4 T cells.

Figure 4. Splenocytes from OAT-4828-treated and CT26-bearing mice have enhanced tumor cells killing capacity



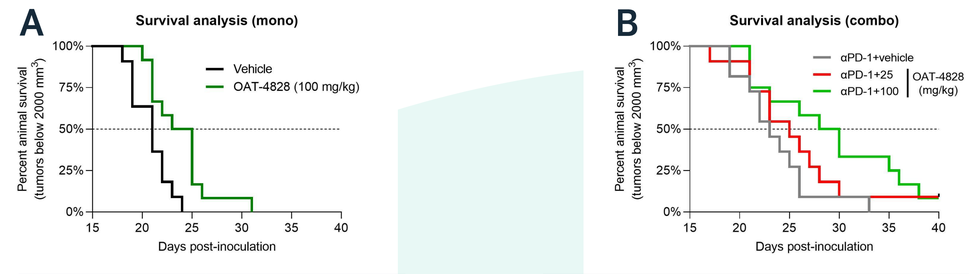
Mice were engrafted with CT26 coloractal cancer cells and treated with different doses of OAT-4828. Isolated splenocytes from CT26-bearing controls and OAT-4828-treated mice (E; effectors) were mixed with fluorescently-labelled CT26 cells (T; targets) in two different ratios and co-cultured for 36h. Cell death of CT26 cells were determined by flow cytometry. We showed that splenocytes from OAT-4828-treated animals had enhanced killing capacity of tumor cells.

Figure 5. Significant tumor size reduction in colorectal cancer model (CT26) after OAT-4828 treatment



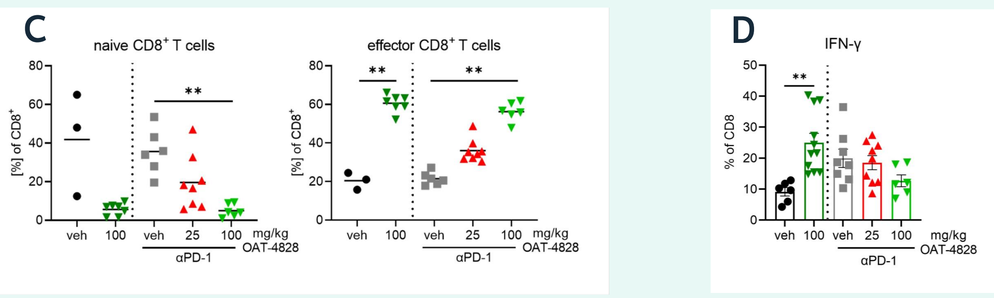
BALB/c mice were inoculated with CT26 cells in the right flank and tumor growth was monitored every 2nd or 3rd day. OAT-4828 was administered twice daily starting from day 2 postinoculation. On day 20 post-inoculation tumors were harvested, weighted and images were taken. OAT-4828 exerts an anti-tumor effect of 38% and 67% reduction in tumor weight for doses 25 and 100 mg/kg, respectively.

Figure 6. OAT-4828 treatment extended survival of mice in monotherapy and in combination with αPD-1 therapy



	Vehicle	OAT-4828 100 mg/kg
Median survival	21 days	24 days
Statistical significance (Mantel-Cox test)		** $p = 0.0059$

	αPD-1	αPD-1 + OAT-4828 (25 mg/kg)	αPD-1 + OAT-4828 (100 mg/kg)
Median survival	23 days	25 days	29 days
Statistical significance (Mantel-Cox test)		ns	* $p = 0.0198$



CT26 cells were inoculated and mice were treated as described in Figure 5. αPD-1 mAb was administed i.p. 4 times every 3rd day starting from day 7 post-inoculation. Mice were culled when tumors reached 2000 mm^3 (pseudosurvival). Flow cytometric analysis was performed on isolated spleens. Survival curve for: **A)** monotherapy with OAT-4828; **B)** in combination with αPD-1 mAb. **C)** Frequency of naive and effector CD8 T cells; **D)** IFN-γ secreted from CD8 T cells after stimulation with PMA/Ionomycin/Brefeldin A for 4h. Mice treated with OAT-4828 had a higher frequency of effector CD8 T cells in spleen which secreted higher levels of IFN-γ in comparison to untreated animals.

Conclusions

Our results showed that OAT-4828 stabilized p53 protein and upregulated p21 in HCT116-treated cells. We verified activity of our USP7 inhibitor in a functional assay of macrophage polarization. Inhibition of USP7 resulted in a dose-dependent reduction of CCL2 expression in M1- and Arg1 in M2-like macrophages.

Additionally, we showed an increase of IFN-γ secretion from activated CD4 T cells treated with OAT-4828. Treatment of tumor-bearing mice with OAT-4828 resulted in an effective and direct killing of CT26 cells *ex vivo* by effector cells isolated from splenocytes in comparison to untreated animals.

Finally, we showed a dose-dependent inhibition of tumor growth in monotherapy with OAT-4828 and in combination with αPD-1 Ab. Splenic CD8 T cells from mice treated with USP7 inhibitor secreted higher levels of IFN-γ and have effector phenotype.

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