# Modulating the tumor microenvironment via hypoxia activated prodrug CPD100Li induced cell death synergistically enhances anti-CTLA-4 immunotherapy

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## Introduction:

The industry is diverting from chemotherapy because of side-effects, primarily immunosuppressive – but not so fast, it could hold immunostimulatory properties. CPD100, a prodrug, is activated in the hypoxic regions of the tumor microenvironment [1, 2]. The liposome formulation, CPD100Li, targets the prodrug to the tumor following the enhanced permeability and retention effect (EPR). Our hypothesis suggests that indiscriminate cell death induced in the hypoxic regions of solid tumors by activating a potent cytotoxin could effectively remodel the tumor microenvironment from tumor directed immunosuppression to modulated immunity under the control of one or more checkpoint molecules. Combining CPD100Li therapy with a checkpoint inhibitor could then unleash activated T cells to kill the cancer selectively.

## Materials and Methods:

1. Immunophenotype Study – CPD100Li
   - Female Balb/c mice (from Envigo) were used for subcutaneous tumor implants of CT26 cells in the right high axilla. Tumor growth was monitored by caliper measurements.
   - Mice were staged when tumors reached 100mm3

| Group | N | Treatment | Dose | ROA | Regimen | Days of Treatment |
|---|---|---|---|---|---|---|
| 1 | 6 | Vehicle | 0.21 ml/20 g | IV | Q7Dx2 | 8, 15 |
| 2 | 6 | Isotype control | 40 mg/kg | IV | Q7Dx2 | 8, 15 |

   - All animals were euthanized by cervical dislocation to prevent introduction of a hypoxic environment.
   - For tumor immune profiling, tumors from all mice were collected 24 hours post last dose, digested to a single cell suspension for flow cytometry (Miltenyi, Germany). CompLeukocyteTM panels were used on an Attune NxT Flow Cytometer (Thermo Fisher Scientific) and analyzed with FlowJo software (Tree Star, Inc. Ashland, OR).

2. Efficacy Study - Combination CPD100Li and Immune Checkpoint Inhibitors
   - Female Balb/c mice (from Envigo) were used for subcutaneous tumor implants of CT26 cells in the right high axilla. Tumor growth was monitored by caliper measurements.
   - Mice were staged into groups of 8 when tumors reached 100mm3.

| Group | Treatment | Dose | ROA | Regimen | Days of Treatment |
|---|---|---|---|---|---|
| 1 | Vehicle | 0.16 ml/20 g | IV | Q14Dx2 | 7, 21 |
| | Isotype control | 10 mg/kg | IP | (Q3Dx2,10)(x2) | 7, 10, 21, 24 |
| 2 | CPD100Li | 30 mg/kg | IV | Q14Dx3 | 7, 21, 35 |
| 3 | CPD100Li | 30 mg/kg | IV | Q14Dx3 | 7, 21, 35 |
| | Isotype control | 10 mg/kg | IP | (Q3Dx2,10)(x2);QDx1(D35) | 7, 10, 21, 24, 35 |
| 4 | Anti-mCTLA-4 | 10 mg/kg | IP | (Q3Dx2,10)(x2) | 7, 10, 21, 24 |
| 5 | Anti-mPD-L1 | 10 mg/kg | IP | Q3Dx2; QDx1(D21) | 7, 10, 21 |
| 6 | Anti-mVISTA | 10 mg/kg | IP | Q3Dx2; QDx1(D21) | 7, 10, 21 |
| 7 | CPD100Li | 30 mg/kg | IV | Q14Dx3 | 7, 21, 35 |
| | Anti-CTLA-4 | 10 mg/kg | IP | (Q3Dx2,10)(x2);QDx1(D35) | 7, 10, 21, 24, 35 |
| 8 | CPD100Li | 30 mg/kg | IV | Q14Dx2 | 7, 21 |
| | Anti-mPD-L1 | 10 mg/kg | IP | Q3Dx2; QDx1(D21) | 7, 10, 21 |
| 9 | CPD100Li | 30 mg/kg | IV | Q14Dx2 | 7, 21 |
| | Anti-mVISTA | 10 mg/kg | IP | Q3Dx2; QDx1(D21) | 7, 10, 21 |

   - Checkpoint inhibitor antibodies were from BioXCell (West Lebanon, NH); Isotype control Clone MPC-11, anti-mCTLA-4 Clone 9D9, anti-mPD-L1 Clone 10F.9G2, anti-VISTA Clone 13F3.
   - Disease progression was monitored by Tumor, Body Weight measurements 3x/week, Increased Time to Progression (ITP) Complete Regression (CR) and Tumor Free Survivors (TFS

## Results:

Flow cytometry demonstrated CPD100Li decreased cell viability in the tumors compared to Vehicle and triggered an increase in the percentage of CD45+ among total live cells (Figure 1). CPD100Li treatment also led to decreased absolute cell counts of Tregs, M-MDSC and M2 macrophages, compared to Vehicle but maintained the percentage of M1 macrophages (Figure 2). Furthermore, the ratio of M1/M2 tumor-associated macrophages increased, suggesting that CPD100Li enhances the persistence of immune cells in the tumor (Figure 3). Finally, a reduction in the expression of PD-1 exhaustion marker on CD8+ T cells was observed (Figure 4). None of the checkpoint inhibitors alone demonstrated a strong response to delaying tumor growth whereas the combination of CPD100Li and CTLA-4 showed a significant synergistic response delaying tumor growth – an increase in time to progression of >200%, and a >60% incidence of complete tumor regressions with 25% remaining as tumor-free survivors at the end of the study (Figure 5).

## Conclusions:

Taken together, these data provide evidence that CPD100Li inhibits CT26 tumor growth by attenuating T cell exhaustion, possibly by a mechanism that promotes M1 macrophage activity. Feasibly, that CPD100Li induces changes in the tumor microenvironment that can convert “cold” tumors into “hot”, promoting a strong anti-tumor response in combination with CTLA-4. This study demonstrates a novel and effective combination strategy for cancer immunotherapy, making CPD100Li a potent candidate for cancer therapy in the near future.

**Figure 1.** A) Mean body weight, B) Mean Tumor volume, C) Live cells (Zombie Viability Dye) as a percentage of total cells and D) CD45 positive cells as a percentage of live cells

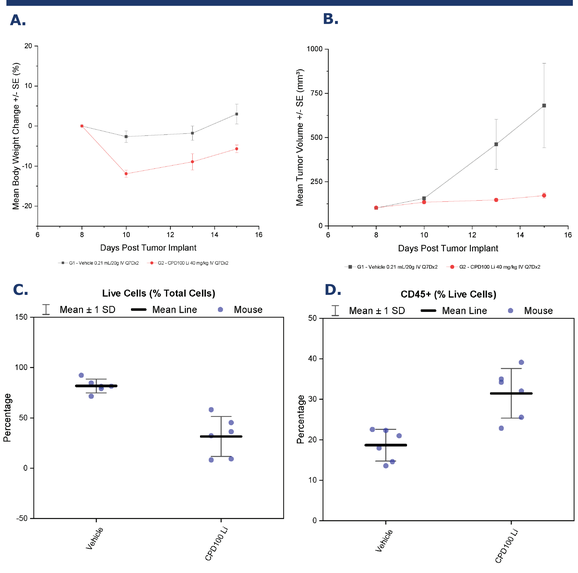



**Figure 2.** Absolute cell counts A) Treg, B) M2 Macrophage and C) M-MDSC

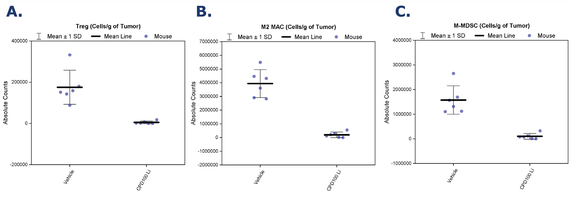



**Figure 3.** Ratio M1/M2 Macrophage absolute cell counts

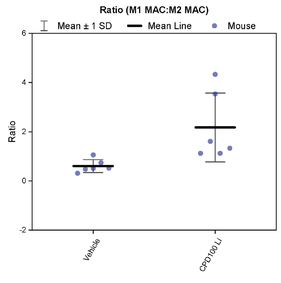



**Figure 4.** PD-1+ cells as a percentage of CD8+ T cells

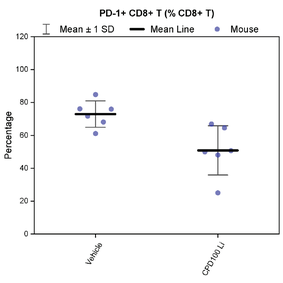
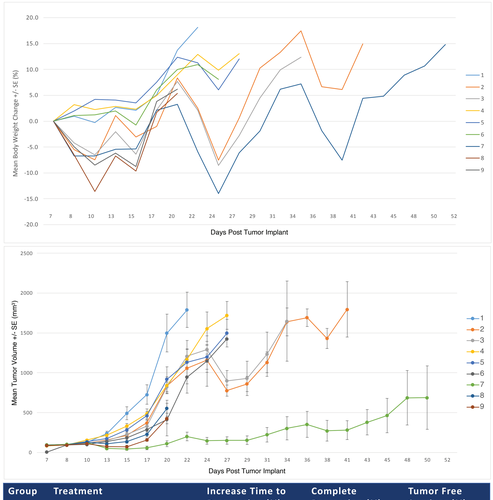



**Figure 5.** A) Body weight, B) Tumor growth, C) Disease Progression Endpoints; Increased Time to Progression (ITP), Complete Regression (CR), Tumor Free Survivors (TFS)




C.

| Group | Treatment | Increase Time to Progression (%) | Complete Regression (%) | Tumor Free Survivor (%) |
|---|---|---|---|---|
| 1 | Vehicle + Isotype Control | NA | 0.0 | 0.0 |
| 2 | CPD100Li | 128 | 0.0 | 0.0 |
| 3 | CPD100Li + Isotype Control | 71 | 0.0 | 0.0 |
| 4 | Anti-mCTLA-4 | 35 | 0.0 | 0.0 |
| 5 | Anti-mPD-L1 | 42 | 0.0 | 0.0 |
| 6 | Anti-mVISTA | 21 | 0.0 | 0.0 |
| 7 | CPD100Li + anti-mCTLA-4 | >207 | 62.5 | 25.0 |
| 8 | CPD100Li + anti-mPD-L1 | 7 | 0.0 | 0.0 |
| 9 | CPD100Li + anti-mVISTA | 7 | 0.0 | 0.0 |

**References**

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