TARGETING PD-L1 IN COLON CANCER USING NUCLEAR RECEPTOR 4A1 (NR4A1) ANTAGONISTS

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INTRODUCTION

Colorectal cancer (CRC) has been identified as the third most diagnosed type of cancer and the third leading cause of cancer-related deaths in the United States (1). Although various screening tests and treatments are available, some are not effective for detecting patients with advanced stages of CRC (2). Therapeutic regimens for treatment of CRC are highly variable and depend on the tumor type and stage.

The orphan nuclear receptors NR4A1, NR4A2, and NR4A3 are immediate early genes induced by multiple stressors, and the NR4A receptors play an important role in maintaining cellular homeostasis and in pathophysiology. There is increasing evidence that these receptors are involved in important pathways in metabolic, cardio-vascular, and neurologic functions as well as in immune functions and cancer. Studies in this laboratory have identified and characterized the orphan nuclear receptor 4A1 (NR4A1, Nur77) as a novel drug target in hormone dependent (i.e breast) cancer and other solid tumors including lung, pancreatic, colon and kidney cancers and rhabdomyosarcoma (3-5). Results of RNA interference (RNAi) studies demonstrate that NR4A1 is pro-oncogenic and regulates several genes/pathways that are important for cancer cell proliferation, survival and migration/invasion. Treatment with 1,1-bis (3'-indolyl)-1-4-hydroxyphenyl methane (CDIM-8) and related NR4A1 antagonists inhibit expression of NR4A1 and NR4A1/Sp-regulated genes. The potency of CDIM8 and its 4-carboxymethylphenyl analog as inhibitors of tumor growth in vivo is in the 20-40 mg/kg/d range and pharmacokinetic studies for CDIM8 show that this compound is rapidly metabolized (conjugated). In contrast "buttressed analog" of CDIM8 such as the 3'-chloro-5-methoxy analog also inhibited mammary tumor growth and enhanced immune surveillance in a syngeneic mouse model at a dose of 2.5 mg/kg/d. NR4A1/Sp-dependent gene regulation represents a novel pathway of gene regulation by activating genes thrsough interactions with Sp transcription factors bound to their cognate GC-rich promoter elements (6). The PD-L1 gene also contains a proximal GC-rich promoter sequence and recent studies in human gastric cancer cells demonstrate that PD-L1 is a Sp1-regulated gene (7). We hypothesized that PD-L1 may also be an NR4A1/Sp regulated gene that can be targeted by C-DIM/NR4A1 antagonists and thereby mimic the effects of checkpoint inhibitor antibodies. Checkpoint inhibitors represent a relatively new and effective breakthrsough in cancer therapy where the blockade of cancer cell derived PD-L1 from interacting with PD-1 on T-cells reactivates immune surveillance of tumors.

MATERIALS AND METHODS

Cell Lines: SW480, RKO and MC-38 were maintained in DMEM growth medium supplemented with 10% FBS at 37˚C in 5% CO_2 and 95% air.

Cell proliferation assay: SW480, RKO and MC-38 cells were treated for 24 hrs with DIM series of compounds. MTT was added to cells. After 3 hrs, Dimethyl sulfoxide (DMSO) was added, and absorbance was measured at 570 nM.

Western Blots: SW480, RKO and MC-38 were treated for 24 hrs with DIM series of compounds including 3,5-disubstitutedphenyl analogs. Cells were then lysed, and lysates were resolved in 10% SDS-PAGE gels. Proteins were transferred onto PVDF membrane and incubated with primary and secondary antibodies and detected using ECL reagent.

RNA Interference: SW480, RKO and MC-38 were seeded in six-well plates and allowed to grow to 60% confluence, then transfections were performed with Lipofectamine 2000 according to the manufacturer's protocol. Both siNR4A1 oligonucleotides and nontargeted control small interfering RNAs were used. Six hrs after transfection, the medium was replaced and left for 72 hrs. The cells were harvested, and protein expression was determined.

ChIP assay: the cells were treated with DIM-3-Br-OCF3 and subjected to ChIP analysis using the ChIP-IT Express magnetic chrsomatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol

RESULTS

1. **NR4A1 inactivation inhibits PD-L1 expression:** Fig.1 show that transient knockdown of NR4A1 with two oligonucleotides siNR4A1 (1) and siNR4A1 (2) in SW480, RKO and MC-38 cells significantly decreased PD-L1 expression (Fig.1A) whereas there is no change in NR4A1 expression after transient knockdown of PD-L1 with two oligonucleotides siPD-L1 (1) and siPD-L1 (2) (Fig.1B).

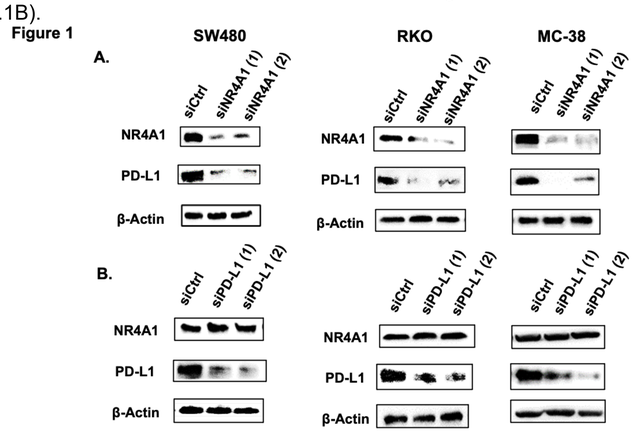

Figure 1. SW480, RKO and MC-38 cells were transfected with siCtrl (non-specific oligonucleotide) and two oligonucleotides targeting NR4A1 (siNR4A1(1) and siNR4A1(2)) or PD-L1 (siPD-L1(1) and siPD-L1(2)) for 72 hrss. Protein expression from whole cell lysates were analyzed by western blots and effects on PD-L1 expression were determined.

2. **Sp1 inactivation inhibits PD-L1 expression:** Fig.2 illustrates that transient knockdown of SP1 with two oligonucleotides siSP1 (1) and siSP1 (2) in SW480, RKO and MC-38 cells significantly decreased expression of PD-L1 (Fig.2A). Also, treatment with mithrsamycin (150 and 300nM) a drug that blocks Sp-mediated transactivation significantly decreased expression of both in these cells resulted in significant decrease of both SP1 and PD-L1 expression (Fig.2B).

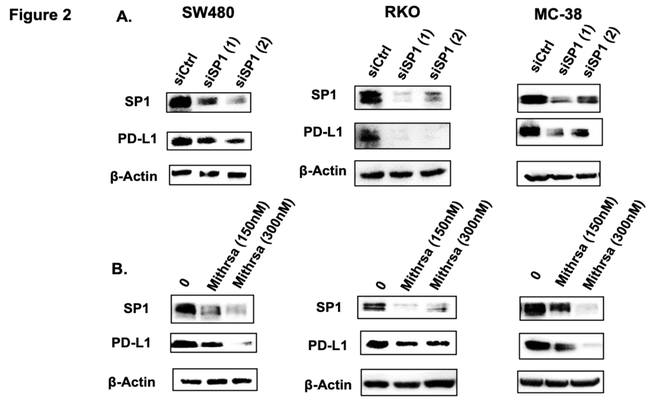

Figure 2. SW480, RKO and MC-38 cells were transfected with siCtrl and oligonucleotides targeting Sp1 (siSp1(1) and siSp1(2)) for 72 hrs as well as treated with Mithrsamycin (150 and 300 nM) for 24 hrs. Protein expression from was analyzed by western blots and effects on PD-L1 levels were determined.

3. **Inactivation of NR4A1 inhibits cell proliferation:** Treatment of SW480, RKO and MC-38 cells with various 3,5-disubstituted phenyl DIM and analog significantly inhibited cell growth (Fig.3A- 3C).

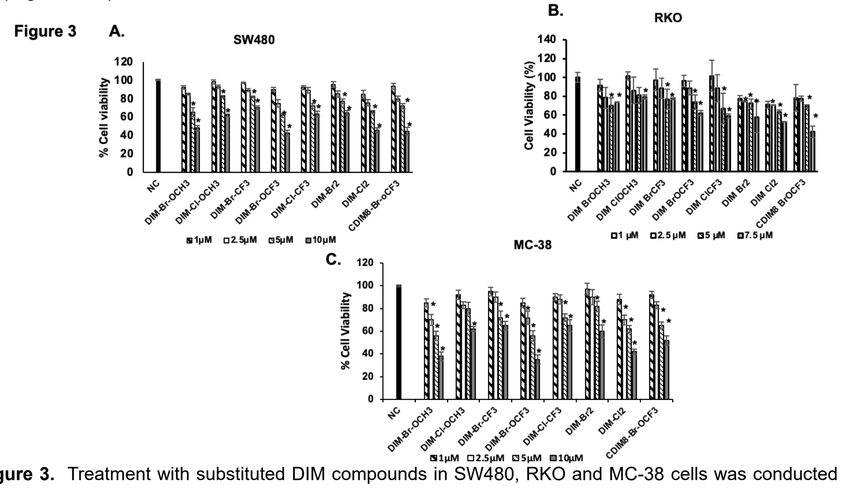

Figure 3. Treatment with substituted DIM compounds in SW480, RKO and MC-38 cells was conducted and effects on cell proliferation were observed. Results are expressed as means ± SD for at least 3 replicate determinations for each treatment group and significant ($P<0.05$) inhibition is indicated (*).

RESULTS

4. **DIM analogs inhibited PD-L1 expression thrsough NR4A1:** Treatment with the same set of DIM compounds inhibited PD-L1 protein levels and the potent DIM-3-Br-5-OCF3 analog inhibited PD-L1 expression in a dose dependent manner in the 3 colon cancer cell lines (Fig. 4B)

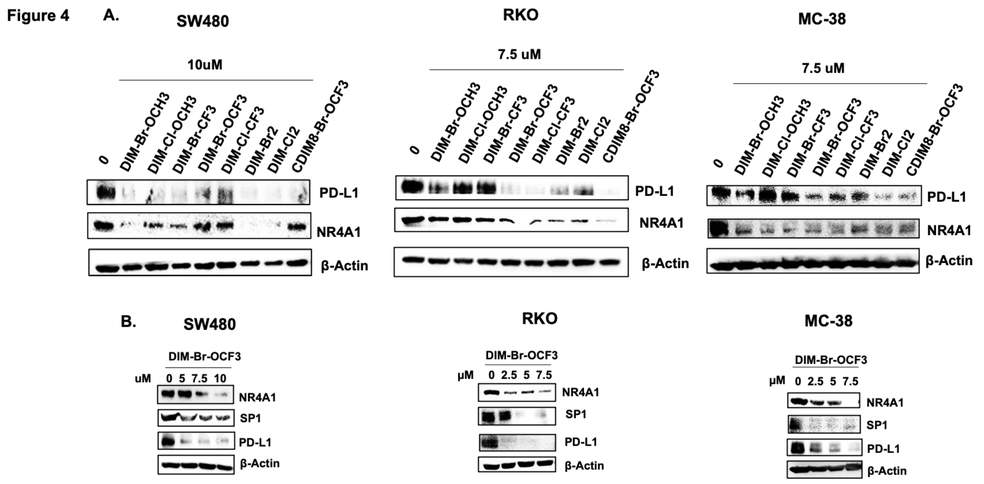

Figure 4. SW480, RKO and MC-38 cells were treated with substituted DIM compounds for 24 hrss and the levels of NR4A1 and PD-L1 protein expression were determined in whole cell lysates by western blot analysis. The dose dependent effects of DIM-3-Br-5-OCF_3 with these cells were performed and the levels of NR4A1 and PD-L1 protein expression were determined.

5. **Role of NR4A1/Sp in regulation of PD-L1.** In SW480, RKO and MC-38 cells ChIP assay was used to detect interactions of NR4A1, Sp1 and pol II with the proximal GC-rich region of the PD-L1 promoter. After treatment with DIM-3-Br-5-OCF3 interactions of these proteins with the GC-rich PD-L1 promoter region was decreased (Fig. 5A, B and 5C) and these data are consistent with the role for the NR4A1/Sp1 complex in regulating PD-L1 expression.

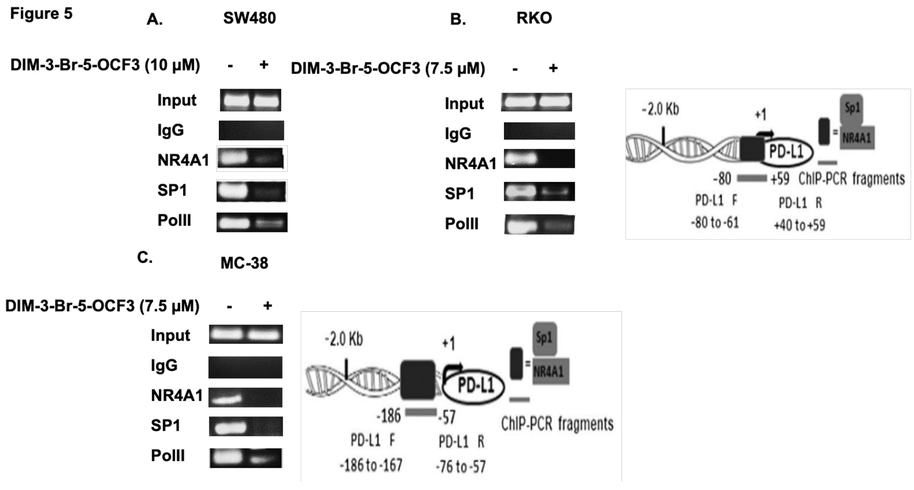

Figure 5. SW480, RKO and MC-38 cells were treated with DIM-3-Br-5-OCF3 for 24 hrss and protein interactions with the GC-rich PD-L1 promoter region were analyzed by ChIP using primers encompassing GC-rich region of the promoter.

SUMMARY AND CONCLUSION

- Bis-indole derived NR4A1 antagonists inhibit PD-L1 expression.
- NR4A1/SP1 regulates PD-L1 and is inhibited by NR4A1 antagonist.
- NR4A1 ligands such as DIM-3-Br-5-OCF_3 were among the most potent of the substituted DIM compounds and ongoing in vivo studies show that this DIM compound also inhibits tumor growth in a syngenic mouse model (data not shown)

Data from this study demonstrate the pro-oncogenic activity of NR4A1 and show that the synthetic buttressed analog DIM-3-Br-5-OCF_3 acts as an NR4A1 antagonist and inhibits PD-L1 expression. These drugs can be developed for future clinical applications.

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