NEW CHECKPOINTS CONTROLLING FUNCTION OF CYTOTOXIC LYMPHOCYTES INFILTRATING HUMAN CARCINOMA

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Background: Although present in high numbers, T and NK cells appear functionally impaired in the renal cell carcinoma (RCC) tumor milieu, as they cannot be stimulated to degranulate and IFN-γ production. This is in part due to altered regulation of signaling downstream of the T cell receptor (TCR). Increased diacylglycerol kinase alpha (DGK-α) has been observed in T and NK cells from the RCC tumor microenvironment (TME). Ex vivo inhibition of DGK-α by the commercially available inhibitor R59022 was able to restore responsiveness to stimulation. Inhibition of DGK-α is reported to also block tumor cell growth and survival.

Many T cells from RCC additionally express the immune checkpoint Programmed cell Death-1 (PD-1). Interaction of PD-1 with PD-L1 on tumor cells blocks AKT signaling and inhibits T cell function. In the clinic, blocking the PD-1/PD-L1 interaction allows tumor control in some patients; however, the majority of patients do not respond long-term. Since DGK-α acts downstream of PD-1 it may, if overactive, curb T cell function despite PD-1/PD-L1 blockade. Thus, we hypothesize that dual inhibition of PD-1 and DGK-α might be required to fully unleash the T cell’s potential in the TME. Current DGK-α inhibitors are not suitable for clinical application. Therefore, we investigate alternative means using RNA interference (RNAi) to target DGK-α alone as well as in combination with PD-1.

Methods: Knockdown was achieved by RNAi using INTASYL™ compounds, developed by Phio Pharmaceuticals. These compounds incorporate drug-like properties into siRNA, resulting in enhanced uptake with no need for transfection reagents. Efficacy was analyzed on mRNA and protein level by rt-qPCR, flow cytometry and Western Blot. Functional assays include cytotoxicity and cytokine production in tumor-mimicking environments.

Results: Using INTASYL™ compounds, silencing of DGK-α was observed in human U2OS osteosarcoma as well as K562 erythroleukemic cells. PD-1 knockdown was achieved in human T cells isolated from peripheral blood mononuclear cells (PBMC). Synergy of DGK-α and PD-1 knockdown is tested in tumor-mimicking in vitro systems using T cell/tumor cell co-cultures at high tumor cell density where T and NK cells become functional suppressed as observed in the TME.

Conclusions: Strong activity of specific T and NK cells is necessary for tumor control. Dual targeting of PD-1 and DGK-α may be required to fully enable T and NK cell reactivity in the TME. Self-delivering RNAi technology represents a promising approach to targeting intracellular immune checkpoints such as DGK-α, in addition to PD-1 inhibition.

REFERENCES
than resveratrol in inhibiting chemotaxis of HL-60 cells and blocking cell cycle of THP-1 and HL-60 cells at G1/S transition. In addition, NBT-167, but not resveratrol, could increase IL-2 production and T cell proliferation stimulated with anti-CD3 and anti-CD28 and synergize with anti-PD-1 antibody to increase IL-2 and IFN-gamma production in co-culture of allogenic T cells and dendritic cells (MLR).

**Conclusions** Our data showed that NBT-167, a dimer of resveratrol, had anticancer and immunomodulatory activities such as modulation of expression of cytokines in immune cells and induction of cancer cell-killling activities of NK and gamma delta T cells. Generally, NBT-167 appeared to have higher activities than resveratrol in modulating immune cells and inhibiting cancer cells. NBT-167 could be a promising cancer immunotherapeutic agent targeting both cancer cells and immune cells.

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**DEVELOPMENT OF IMPROVED SMALL MOLECULE STING AGONISTS SUITABLE FOR SYSTEMIC ADMINISTRATION**


**Background** Stimulator of Interferon Genes (STING) is a major player in the activation of robust innate immune response leading to initiation and enhancement of tumor-specific adaptive immunity. Several clinical and pre-clinical programs have shown that activation of the STING pathway triggers immune-mediated antitumor response. Although vast majority of programs focus on development of analogues of the endogenous STING ligands, their chemical nature and stability often limit their use to local administration. Herein, we present recent results from the development of our selective non-nucleotide, non-macrocyclic, small molecule direct STING agonists, suitable for systemic administration, characterized by improved activity in human immune cells.

**Methods** Binding to recombinant STING protein was examined using RTS, MST, FP and crystallography studies. Phenotypic screen was performed in THP-1 Dual reporter cells. Mouse bone marrow-derived dendritic cells (BMDC) were obtained from C57BL/6 mice and differentiated with mIL-4 and mGM-CSF. STING agonists were administered into BALB/c mice and cytokine release was measured in plasma. Additionally, mice were inoculated with CT26 murine colon carcinoma or EMT6 murine breast carcinoma cells and the compound was administered, followed by the regular tumor growth and body weight monitoring.

**Results** Ryvu’s small-molecule agonists demonstrate strong binding affinity to recombinant STING proteins across all tested species. The compounds bind to all human STING protein variants and trigger pro-inflammatory cytokine release from human immune cells regardless of the STING haplotype. Moreover, new generation of developed agonists show significantly improved binding to human protein as well as in vitro activity on human cells. Systemic, intravenous in vivo administration leads to a dose-dependent upregulation of STING-dependent pro-inflammatory cytokines, which results in a dose-dependent antitumor efficacy observed in CT26 and EMT6 mouse cancer models, leading to complete tumor remissions in all treated animals. Furthermore, observed efficacy is accompanied by development of a lasting immunological response demonstrated by lack of tumor engraftment or a delayed tumor growth in cured animals challenged with repeated inoculation of cancer cells.

**Conclusions** New generation Ryvu’s STING agonists are strong and selective activators of STING-dependent signaling in both mouse and human immune cells promoting anti-tumor immunity. Treatment with Ryvu’s small-molecule STING agonists leads to engagement of the immune system which results in a complete tumor remission and development of immunological memory of the cancer antigens. The compounds show good selectivity and ADME properties enabling development for systemic administration. In addition developed compounds maintain small functional handles amenable to linker attachment making the series suitable for versatile development as single agents, for combinations with immunotherapies or as targeted agents.

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**STING AGONIST-BASED TREATMENT PROMOTES VASCULAR NORMALIZATION AND TERTIARY LYMPHOID STRUCTURE FORMATION IN THE THERAPEUTIC MELANOMA MICROENVIRONMENT**

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**Background** The degree of immune infiltration in tumors, especially CD8+ T cells, greatly impacts patient disease course and response to interventional immunotherapy. Hence, enhancement of TIL prevalence is a preferred clinical endpoint, one that may be achieved via administration of agents that normalize the tumor vasculature (VN) leading to improved immune cell recruitment and/or that induce the development of local tertiary lymphoid structures (TLS) within the tumor microenvironment (TME).

**Methods** Low-dose STING agonist ADU S-100 (5μg/mouse) was delivered intratumorally to established s.c. B16.F10 melanomas on days 10, 14 and 17 post-tumor inoculation under an IACUC-approved protocol. Treated and control, untreated tumors were isolated at various time points to assess transcriptional changes associated with VN and TLS formation via qPCR, with corollary immune cell composition changes determined using flow cytometry and immunofluorescence microscopy. In vitro assays were performed on CD11c+ BMDCs treated with 2.5 μg/mL ADU S-100 (vs PBS control) and associated transcriptional changes analyzed via qPCR or profiled using DNA microarrays. For TCRβ-CDR3 analyses, CDR3 was sequenced from gDNA isolated from enzymatically digested tumors and splenocytes.

**Results** We report that activation of STING within the TME leads to slowed melanoma growth in association with increased production of angiostatic factors including Tnfsf15 (Vegi), Cxcl10 and Angpt1, and TLS inducing factors including Ccl19, Ccl21, Lta, Ltb and Tnfsf14 (Light). Therapeutic responses from intratumoral STING activation were characterized by increased vascular normalization (VN), enhanced tumor infiltration by CD8+ T cells and CD11c+ DCs and local TLS neo-genesis, all of which were dependent on host