Feasibility Studies for small molecule inhibitors targeting Neuropilin-1 inglioma

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Our overall goal is to conduct hit validation and hit-to-lead studies on our new small-molecule Neuropilin 1 (NRP1) antagonists as immune activators for cancer therapy. We have reported that genetic ablation of NRP1 in microglia and macrophages (MG/MP) improves outcomes in mouse models of primary glioma, i.e. decreases tumor volumes, vascularization, and the num- ber of immunosuppressive regulatory T cells (Tregs), and increases the number of proinflamma- tory MG/MPs and infiltration of cytotoxic CD8+ T cells. We have also reported that the prototype molecule EG00229 inhibits Nrp1 *in vivo* and reduces glioma growth when delivered by local in- fusion. Our endpoint in this project will be robust lead molecules suitable for final lead optimisa- tion and clinical candidate selection. We will also optimise our *in vivo* models of primary and metastatic brain tumors**.**

We started by identifying sub-μM peptidomimetic leads that did not have good oral bioavail- ability, and then followed this up by conducting a fragment screen and partly optimizing several hits with good potential for oral bioavailability. By incorporating these drug-like fragments into the design paradigm defined by EG00229, and a follow-up molecule EG01377, we will have generated candidate orally-deliverable, brain-penetrant inhibitors. For our team here at SBU, we now need to experimentally determine their potency, oral absorption and CNS penetration. The following goals will be pursued in this proposal:

Aim 1. Development of Structure-Activity Relationships (SAR), cell toxicity evaluation and blockade of TGFβ signalling with prototype molecules.

Rationale: TGFβ signalling modulates the immune microenvironment at sites of tumor.

Approach: Through fragment screen data and systematic investigation of chemotypes designed to bind in the NRP1 binding pocket, we have identified CNS penetrant, likely orally bioavailable, tight-binding inhibitors. These hits will be validated through resynthesis and detailed analysis (^{1}H, ^{13}C NMR, LCMS). Cell toxicity will be assessed by screening against primary mouse mac- rophages and Tregs, as well as for peripheral blood Treg cells and/or monocytes. The hits will be incubated with T cells, Tregs and MG/MPs and the effects on TGFβ signalling assessed as an immune functional readout, using ELISA assays for TGFβ and immunoblots for the levels of phosphorylated SMAD2/3 (downstream effectors of NRP1/TGFβR activation). These experi- ments will help define and establish the relationships between ligand structure and NRP1 activi- ty in the context of cells of the innate and adoptive immune response.

Aim 2. Development of fragment screen hits with potential for oral absorption and im- mune activity

Rationale: Ensure that the small molecules have good potency and stability prior to *in vivo* ap- plication.

Approach: Hits may be chemically modified according to our knowledge base of NRP1 to in- clude additional binding groups and improve affinity. Dose-response data in Surface Plasmon Resonance (SPR) and competitive fluorescent polarization (FP) assays will be used to triage the compounds. The best modified hits will be screened in *in vitro* absorption, distribution, me- tabolism and excretion (ADME) assays; S9 fraction (mouse, human) and parallel artificial mem- brane permeability assay (PAMPA) will serve as a surrogate for membrane permeability. After determining toxicity *in vivo*, we will establish gliomas in animals, deliver the compounds locally into the tumor or systemically by gavage, and compare

them to the prototype EG00229 by measuring tumor volumes, infiltration of T lymphocytes, abundance of Tregs, and activation states of MG/MP.

Taken together, we aim to identify authentic orally-absorbed and CNS-penetrant small molecule inhibitors of NRP1 with nanomolar potency that can be used as immunomodulators of the tumor microenvironment.