Dose selection investigations and combination strategies of NM21-1480, a PD-L1/4-1BB/HSA trispecific MATCH3 therapeutic clinical candidate

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Concept: Tumor-localized activation of 4-1BB combined with PD-L1 blockade

TUMOR-LOCALIZED ACTIVITY

NM21-1480 leverages monovalent binding to its targets and ultra-high-affinity to PD-L1 (K_D=7E-12M) in order to restrict 4-1BB signaling to the tumor microenvironment (TME), thereby allowing for safe combination of synergistic immune stimulatory activities

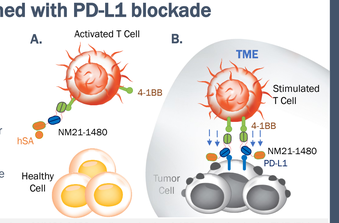


Figure 1. Differentiated molecular design of NM21-1480 to allow selective 4-1BB activation in the TME (B) but not in normal tissue (A). NM21-1480 does not intrinsically trigger 4-1BB clustering and signaling upon binding to 4-1BB alone. Only with simultaneous binding of 4-1BB and PD-L1 can clustering of 4-1BB occur, resulting in 4-1BB signaling and concomitant blocking of the PD-1 / PD-L1 pathway.

NM21-1480 consists of three λcap™ -stabilized antibody Fvs fused in a single chain

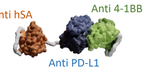


Figure 2. Schematic representation of NM21-1480: a trispecific scMATCH3 molecule with αPD-L1 (VL1/VH1 in blue), α4-1BB (VL2/VH2 in green) and αHSA (VL3/VH3 in red) λcap™ -stabilized antibody Fv regions connected by Gly-Ser peptide linkers in a single polypeptide chain.

Membrane distal 4-1BB binding allows for concomitant 4-1BBL binding

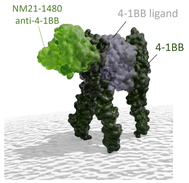
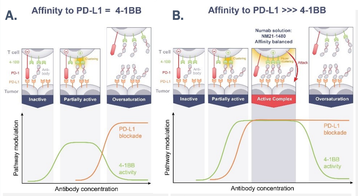


Figure 3. Surface representation of a structural model based on X-ray crystallography complex structures showing the trimer of 4-1BB (extracellular domain; dark green), clustered by its natural trimeric ligand, 4-1BB-L (gray), and the 4-1BB-binding Fv region of NM21-1480 (anti-4-1BB scFv; light green).

Affinity optimization is key for overlapping 4-1BB activation and PD-L1 blockade

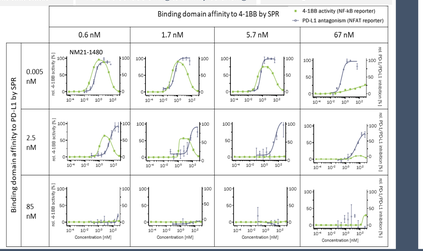


Suboptimal 4-1BB activation at maximal PD-L1 blockade and vice versa

Overlapping, optimal 4-1BB activation and PD-L1 blockade owing to affinity-balancing

Figure 4. Schematic representation of optimization of dual action (4-1BB / PD-L1) through affinity-balancing in NM21-1480
A. With similar affinities for PD-L1 and 4-1BB no drug concentration provides concomitant maximal stimulation of 4-1BB and PD-L1 antagonism.
B. By increasing the affinity to PD-L1 compared to 4-1BB, as in NM21-1480, maximal simultaneous stimulation of 4-1BB and PD-L1 antagonism can be achieved over a broad dose range.

Figure 5. Drug concentration-response curves for 12 scMATCH3 analogues with the indicated α4-1BB and αPD-L1 affinities. The top left panel is data from NM21-1480. Responses were measured from cells containing NF-AT (PD-L1, blue) or NF-κB (4-1BB, green) reporter genes.



Synergistic activity of PD-L1 blockade and 4-1BB signaling is maintained over a broad range of concentrations

- PD-L1 inhibition and 4-1BB stimulation both contribute to activity (efficacy and potency) in a Mixed Lymphocyte Reaction (MLR)
- NM21-1480 significantly increases levels of cytokine release when compared to anti-PD-L1 or anti-4-1BB monoclonal antibodies (data not shown), or the combination of such antibodies (Fig. 6A)
- We did not observe a bell-shaped dose response for TNFα or IFNγ release with NM21-1480 even at concentrations up to 500 nM (Fig. 6B)
- A bell-shaped response was observed for IL-2 release (Fig. 6B), maybe related to the known phenomenon of IL-2 re-uptake by T cells via CD25
- Since such levels of cytokine release are not induced by anti-PD-L1 antibody alone, it appears that the effect of combined 4-1BB stimulation and PD-L1 blockade is maintained at relatively high concentrations with NM21-1480

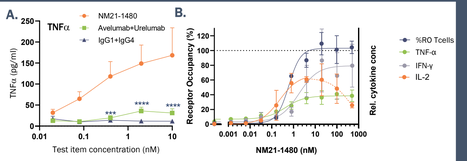


Figure 6. Monocyte derived dendritic cells (MoDCs) were prepared from CD14+ cells cultured for 7 days. MoDCs were then cultured together with T cells from a separate donor for 5 days in the presence of a dose titration of NM21-1480, avelumab, urelumab, IgG1 + IgG4. Supernatants and cells were collected at the end of culture (or 48hrs for IL2) and cytokine production was measured by ELISA and receptor occupancy of NM21-1480 on T cells was measured by flow cytometry. **A.** NM21-1480 induces significantly greater TNF-α release than the combination of avelumab and urelumab. **B.** NM21-1480 induces plateauing release of TNF-α and IFN-γ, and bell-shaped release of IL-2.

In vivo dose-response relationship in a syngeneic mouse tumor model demonstrates maintained 4-1BB stimulation and complete tumor regression at high doses, and the formation of immunological memory.

We have tested the dose-response relationship of PRO1601 in a triple knock-in huPD-L1, huPD-1, hu4-1BB mouse with implanted huPD-L1.MC38 syngeneic tumors. PRO1601 is a mouse surrogate for NM21-1480 with a mouse cross-reactive HSA binder. At doses of 16.5 and 50 mg/kg every 5 days complete tumor regression was observed, and the formation of immunological memory was induced. The PRO1601 dose-dependent increase of 4-1BB stimulation specific markers was maintained up to the highest dose tested, indicating optimal dual activation at least up to these dose levels.

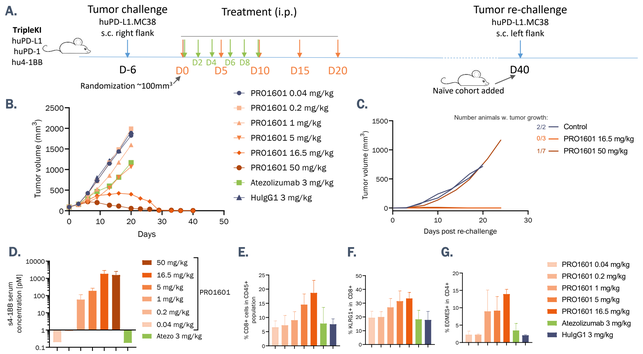


Figure 7. A. Triple knock-in (KI) transgenic mice expressing human PD-L1, PD-1 and 4-1BB were inoculated with huPD-L1 expressing MC38 cells subcutaneously (s.c.) in the right flank. Six days later the mice were randomised into 8 groups and mice were dosed intraperitoneally (i.p.) with PRO1601 from 0.04 mg/kg to 50 mg/kg every 5 days for 5 doses (orange arrows), huIgG1 negative control or atezolizumab (anti-PD-L1) at 3 mg/kg every 2 days for 6 doses (green arrows). After 40 days, mice without remaining measurable tumor from dose groups PRO1601 at 16.5 mg/kg and 50 mg/kg were re-challenged with huPDL1.MC38 cells s.c. in the left flank. Two naive control mice were also rechallenged with huPDL1.MC38 cells. **B.** Mean tumor volume is plotted per cohort versus time post first dose. Tumor growth inhibition was observed for mice treated with PRO1601 at 1 and 5 mg/kg and atezolizumab at 3 mg/kg. Tumor regression was observed for mice treated with 16.5 and 50 mg/kg. **C.** Tumor growth was measured from mice rechallenged with PD-L1.MC38 cells. 3/3 and 6/7 mice from 16.5 and 50 mg/kg groups respectively had no tumor regrowth following rechallenge. **D.** Serum was collected from mice 2 hours after the last dose of test item and soluble 4-1BB was measured by ELISA as a marker of 4-1BB-specific T cell activation.

Tumors were collected at the end of the main study and processed to single cell suspension for flow cytometry. Data indicate a PRO1601 dose-dependent increase in T cell activation, including **E.** % CD8+ cells in TIL population; **F.** %KLRG1-positive CD8 cells; **G.** % EOMES-positive CD4 cells

In vivo dose response relationship in a syngeneic mouse tumor model demonstrates a bell-shaped dose response for tumor growth inhibition and 4-1BB stimulation with increased drug exposure (dose and frequency).

In order to try to observe an *in vivo* bell-shaped dose response we increased the exposure of the animals to PRO1601 by increasing the dose of PRO1601 to 100 mg/kg and increased the dosing frequency to every two days. In this experiment a bell-shaped dose response was observed, with maximal activity seen at 10 mg/kg, which matched a concomitant bell-shaped response in levels of soluble 4-1BB, a marker of 4-1BB-specific stimulation[1].

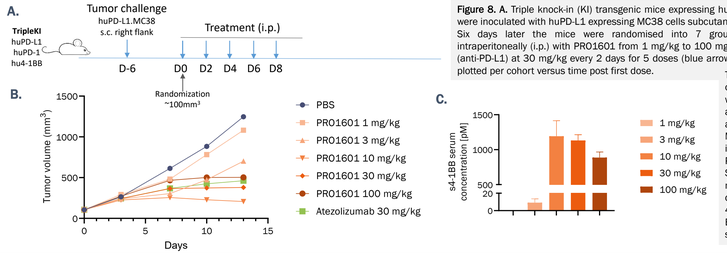
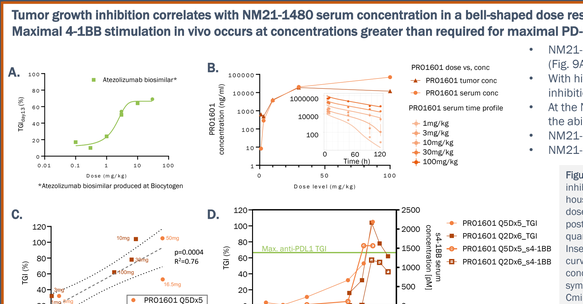


Figure 8. A. Triple knock-in (KI) transgenic mice expressing human PD-L1, PD-1 and 4-1BB were inoculated with huPD-L1 expressing MC38 cells subcutaneously (s.c.) in the right flank. Six days later the mice were randomised into 7 groups and mice were dosed intraperitoneally (i.p.) with PRO1601 from 1 mg/kg to 100 mg/kg, vehicle or atezolizumab (anti-PD-L1) at 30 mg/kg every 2 days for 5 doses (blue arrows). **B.** Mean tumor volume is plotted per cohort versus time post first dose.

Tumor growth inhibition was observed for mice treated with PRO1601 at 3, 10, 30 and 100 mg/kg and atezolizumab at 30 mg/kg. Maximum tumor growth inhibition was observed with PRO1601 at 10 mg/kg. **C.** Serum was collected from mice 48 hours after the last dose of test item and soluble 4-1BB was measured by ELISA as a marker of 4-1BB-specific T cell activation[1].

Tumor growth inhibition correlates with NM21-1480 serum concentration in a bell-shaped dose response and correlates with the 4-1BB-specific activation marker, soluble 4-1BB[1]. Maximal 4-1BB stimulation in vivo occurs at concentrations greater than required for maximal PD-L1 blockade, allowing for concomitant maximal 4-1BB stimulation and PD-L1 inhibition.



- NM21-1480 in vivo efficacy is greater than that observed with saturating doses of anti-PD-L1 therapy alone (Fig. 9A and D) and correlates with 4-1BB stimulation (Fig. 9C).
- With higher drug concentrations, 4-1BB specific activity decreases as demonstrated by reduced tumor growth inhibition and reduced soluble 4-1BB (Fig. 9D), replicating the bell-shaped dose-response seen in vitro (Fig. 5).
- At the NM21-1480 exposure of maximum 4-1BB activation, maximum anti-PD-L1 activity is reached indicating the ability to concomitantly achieve 100% PD-L1 blockade and 4-1BB stimulation (Fig. 9D).
- NM21-1480 shows a non-linear pharmacokinetic profile indicative of target mediated drug disposition (TMDD)
- NM21-1480 penetrates into the tumor to similar concentrations as present within the serum (Fig. 9B).

Figure 9. Integration of the in vivo data. **A.** Tumor growth inhibition activity of anti-PD-L1 atezolizumab (Biocytogen in-house) shows maximal activity at 10-30 mg/kg. **B.** PRO1601 dose-concentration relationship in tumor and serum at 120hr post single dose. Tumor concentrations were measured by quantitative mass spectrometry of collected tumor material. Inset: serum concentration-time profile and TMDD PK fitting curve. **C.** The tumor growth inhibition* versus soluble 4-1BB concentrations in serum. **D.** Tumor growth inhibition* (closed symbol) and soluble 4-1BB in serum (open symbol) versus Cmin (trough) levels of PRO1601 post first dose.
*To account for different dosing regimens in vivo studies, TGI is based on the growth rate estimation between day 3 and day 13.

NM21-1480 is also efficacious in models of immunologically "cold" tumors

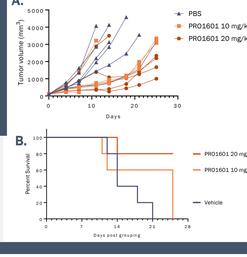


Figure 10. Triple Knock-in transgenic mice expressing human PD-L1, PD-1 and 4-1BB were inoculated with human PD-L1 expressing B16F10 cells subcutaneously (s.c.) in the right flank. When the tumors reached approx.100 mm³, the mice were randomized and treated with PBS or PRO1601 (10mg/kg and 20 mg/kg, respectively) via i.p administration, every 5 day for a total of 3 doses. The tumor volumes were measured twice per week for a total of 32 days (**A.**). The survival curves were plotted with survival rate versus days post grouping (**B.**).

The combination of NM21-1480 and CD3-T cell engager has the potential to form a super-agonist synapse for enhanced T cell activation

Targeted modulation of the tumor microenvironment by combining T cell recruiting T cell engager NM32-2668 with the next generation checkpoint inhibitor NM21-1480 to form a super-agonistic immunological synapse.

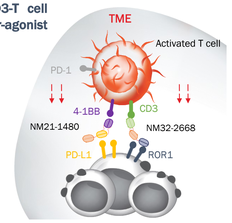


Figure 11. NM21-1480 and the ROR1 targeting molecule NM32-2668 (ROR1.CD3.hSA) synergize to increase T cell activation and to improve the impact on tumor growth and eradication.

NM21-1480 enhances the in vivo anti-tumor efficacy of a tumor targeted CD3 T cell engager and increases memory populations of tumor infiltrated lymphocytes

Figure 13. NCG mice were injected subcutaneously (s.c.) with a mixed implant of 1x10⁷ JIMT-1 tumor cells and 5 x 10⁶ PBMCs in 50% Matrigel. Five days later, animals were dosed and subsequently every 5 days with test item intravenously (i.v.). **A.** Mean tumor volume is plotted per cohort versus time.
Tumors were collected at the end of the study and processed to single cell suspension for flow cytometry. **B.** CD8 effector memory (CCR7-CD45RO+) cell counts per mg tumor and **C.** CD8 central memory (CCR7+CD45RO+) cell counts per mg tumor are plotted per cohort.

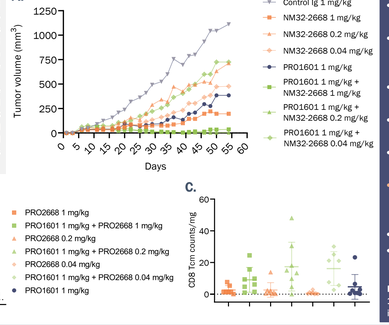
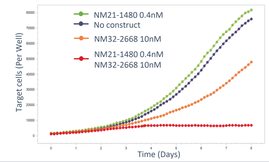


Figure 12. The combination of NM21-1480 with the T cell engager NM32-2668, significantly increases the cytolytic activity against tumor antigen positive target cells. JIMT-1 tumor cells were cocultured with T cells for up to 8 days at an E:T ratio of 5:1 in the presence of NM21-1480 or the ROR1 T cell engager NM32-2668, or a combination of the two. JIMT-1 cells were labeled with NucLight Red cell dye and imaged at regular intervals using the IncuCyte, a real time live cell imaging platform. The y-axis depicts the numbers of target cells normalized to time 0. The no construct control demonstrated tumor cell growth in the absence of treatment.



Conclusions

- **NM21-1480 is a highly potent, affinity optimized, multi-specific PD-L1 antagonist and 4-1BB agonist molecule currently in Phase I clinical development**
- **Optimized affinity levels for PD-L1 and 4-1BB binding assures an overlap in optimal concentration for 4-1BB agonism and PD-L1 antagonism and extends the window for maximal dual activity**
- **Significantly greater efficacy in a mixed lymphocyte reaction compared to a mixture of anti-PD-L1 and anti-4-1BB antibodies, with sustained activity at high doses**
- **Potent activity observed in vivo with tumor regression and immunological memory formation demonstrated**
- **Tumor growth inhibition of NM21-1480 correlates with 4-1BB specific activation markers in vivo and a bell-shaped dose response is observed for 4-1BB agonism**
- **Maximal 4-1BB stimulation in vivo occurs at concentrations greater than required for maximal PD-L1 blockade, allowing for concomitant maximal 4-1BB stimulation and PD-L1 blockade**
- **In vivo activity is also observed in an immunologically "cold" tumor model**
- **Combination with a CD3 T cell engager shows enhanced activity in vitro and in vivo, with increased anti-tumor activity and CD8+ memory cells within the tumor**

References:
1. Glez-Vaz J, Azpilikueta A, Olivera I, et al. Soluble CD137 as a dynamic biomarker to monitor agonist CD137 immunotherapies. Journal for ImmunoTherapy of Cancer 2022;10:e003532. doi:10.1136/jitc-2021-003532