TGFβR1 Blockade with Galunisertib (LY2157299) Enhances Anti-Neuroblastoma Activity of the Anti-GD2 Antibody Dinutuximab (ch14.18) with Natural Killer Cells

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Abstract

Purpose: Immunotherapy of high-risk neuroblastoma using the anti-GD2 antibody dinutuximab induces antibody-dependent cell-mediated cytotoxicity (ADCC). Galunisertib, an inhibitor of TGFβR1, was examined for its ability to enhance the efficacy of dinutuximab in combination with human ex vivo activated NK (aNK) cells against neuroblastoma.

Experimental Design: TGFβ1 and TGFβR1 mRNA expression was determined for 249 primary neuroblastoma tumors by microarray analysis. The ability of galunisertib to inhibit SMAD activity induced by neuroblastoma patient blood and bone marrow plasma in neuroblastoma cells was tested. The impact of galunisertib on TGFβ1-induced inhibition of aNK cytotoxicity and ADCC in vitro and on anti-neuroblastoma activity in NOD-scid gamma (NSG) mice was determined.

Results: Neuroblastomas express TGFβ1 and TGFβR1 mRNA. Galunisertib suppressed SMAD activation in neuroblastoma cells induced by exogenous TGFβ1 or by patient blood and bone marrow plasma, and suppressed SMAD2 phosphorylation in human neuroblastoma cells growing in NSG mice. In NK cells treated in vitro with exogenous TGFβ1, galunisertib suppressed SMAD2 phosphorylation and restored the expression of DNAM-1, Nkp30, and NKG2D cytotoxicity receptors and the TRAIL death ligand, the release of perforin and granzyme A, and the direct cytotoxicity and ADCC of aNK cells against neuroblastoma cells. Addition of galunisertib to adoptive cell therapy with aNK cells plus dinutuximab reduced tumor growth and increased survival of mice injected with two neuroblastoma cell lines or a patient-derived xenograft.

Conclusions: Galunisertib suppresses activation of SMAD2 in neuroblastomas and aNK cells, restores NK cytotoxic mechanisms, and increases the efficacy of dinutuximab with aNK cells against neuroblastoma tumors. Clin Cancer Res; 23(3): 804–13. ©2016 AACR.

See related commentary by Zenarruzabeitia et al., p. 615

Introduction

High-risk neuroblastoma accounts for a disproportionate burden of childhood cancer morbidity and mortality, representing 7% of childhood malignancies but accounting for 15% of all childhood cancer-related deaths (1). Although event-free survival for patients with high-risk neuroblastoma has improved with use of the anti-disialoganglioside (anti-GD2) chimeric mAb dinutuximab (ch14.18) plus IL2 and GM-CSF immunotherapy, 40% of patients still relapse during or after immunotherapy (2). The reasons for failure of this immunotherapy are not known.

Increasing evidence indicates that the tumor microenvironment (TME) supports tumor growth and survival and regulates immune responses (3). Within the TME, the TGFβ family has an important role in tumor immune evasion, leading to tumor progression and metastasis (4, 5). The clinical importance of TGFβ1 in suppressing NK cells is indicated by two studies of patients with breast or squamous cell carcinoma (6, 7). Among the TGFβ family, TGFβ1 is the most commonly upregulated in tumor cells (5, 8). This ligand binds to TGFβ receptor type I (TGFβR1), which results in its dimerization to TGFβ receptor type II (TGFβR2). This heterodimer then phosphorylates SMAD2 and SMAD3, which complex with SMAD4 to modulate transcription of downstream genes (9, 10). TGFβ1 is known to inhibit the IFNγ production, proliferation, and function of natural killer (NK) cells, an important type of immune effector cell expressing the antibody receptor FcγRIIa (CD16) and mediating ADCC against neuroblastoma cells (11–13). The neuroblastoma TME can include TGFβ1, and higher than median TGFβ1 in MYCN non-amplified neuroblastoma patient tumors correlates with worse event-free survival (14).
Translational Relevance

Anticancer functions of natural killer (NK) cells may be suppressed in the tumor microenvironment. The cytokine TGFβ1 can be a major effector of this suppression. Galunisertib, a small-molecule inhibitor of TGFβRI, enhances antitumor activity when combined with paclitaxel or sorafenib in xenograft models of breast or hepatocellular carcinoma and is being tested clinically. However, the effect of galunisertib on TGFβ suppression of NK cell function has not been investigated. We demonstrate that galunisertib reverses TGFβ1-induced suppression of direct cytotoxicity and anti-GD2 antibody-dependent–cell–mediated cytotoxicity of human ex vivo propagated and activated NK (aNK) cells against neuroblastoma cells in vitro. Furthermore, galunisertib enhances aNK adoptive cell therapy with the anti-GD2 monoclonal antibody dinutuximab against neuroblastoma cell lines and a neuroblastoma patient-derived xenograft growing in NSG mice. These data suggest that galunisertib may improve anti-GD2 antibody-based immunotherapy of neuroblastoma.

Approaches for inhibiting TGFβ–induced signaling include targeting ligand–receptor interactions and intracellular signaling (15). Galunisertib (LY2157299 monohydrate) is a recently developed small-molecule inhibitor of TGFβRI. Galunisertib binds antagonistically to TGFβRI, preventing the intracellular phosphorylation of SMAD2 and SMAD3 (16–18). This agent has demonstrated antitumor activity in combination with paclitaxel or sorafenib in xenograft models of breast or hepatocellular carcinoma (17–19). Phase I studies have demonstrated that galunisertib is safe in adult patients with advanced solid tumors (20, 21). However, it is unknown whether galunisertib can augment anti-GD2 antibody therapy or the antitumor cytotoxicity of NK cells propagated and activated ex vivo with K562.mbIL21 artificial antigen-presenting cells (22–24), which we and others are using to generate activated NK (aNK) cells for evaluation in clinical trials of adoptive cell therapy (ClinicalTrials.gov identifiers: NCT01787474 and NCT02573896).

We demonstrate that galunisertib significantly restores the cytotoxicity of aNK cells following their inhibition by TGFβ1 in vitro and enhances the combination of dinutuximab and aNK cell immunotherapy against neuroblastoma cell lines and a patient-derived xenograft (PDX) growing in kidneys of NOD-scid gamma (NSG) mice. These findings support the clinical testing of galunisertib in combination with dinutuximab for the immunotherapy of neuroblastoma.

Materials and Methods

Neuroblastoma cells, patient specimens, aNK cells, and reagents

CHLA-255 and CHLA-136 neuroblastoma cell lines were maintained in Iscove’s modified Dulbecco’s medium (IMDM) with 10% FBS. CHLA-255–Fluc and CHLA-136–Fluc cells were transduced with the firefly luciferase (Fluc) gene using a lentivirus vector (24). COGN-N-415x PDX neuroblastoma cells expressing mutated ALK (F1174I) and amplified MYCN gene were kindly provided by Dr. C. Patrick Reynolds (Texas Tech University, Lubbock, Texas). The correct identity of cells was authenticated using the AmpFLSTR Identifier PCR Amplification Kit (Applied Biosystems). Primary neuroblastoma tumors were obtained from patients enrolled and consented for Children’s Oncology Group (COG) biology and therapeutic protocols. Plasma from whole blood and bone marrow aspirates were obtained from patients with relapsed and refractory neuroblastoma enrolled on the New Approaches to Neuroblastoma Therapy (NANT) Biology Study N2004-05.

NK cells from healthy donors were activated and propagated ex vivo using K562.mbIL21 artificial antigen-presenting cells (22–24) genetically engineered to express immunostimulatory molecules, including CD137 ligand and membrane-bound IL21 (K562.mbIL21), the latter of which was associated with increased telomere length in cultured NK cells (24). In brief, peripheral blood mononuclear cells (PBMC) were coincubated at day 0 with irradiated (100 Gy) K562.mbIL21 cells at a ratio of 2:1 in NK-cell expansion medium (NKEIM) comprised of RPMI1640 and 10% FBS with 50 IU/mL recombinant human IL2 (PeproTech). On day 7, cultures were replenished with irradiated K562.mbIL21 cells and fresh NKEIM. aNK cells were then viably frozen at day 14 in 50% Cryoprotective Medium (Lonza), 25% RPMI1640, and 25% FBS.

Anti-GD2 chimeric monoclonal antibody (mAb) ch14.18/dinutuximab was provided by the National Cancer Institute (Frederick, MD). Human TGFβ1 (R&D Systems) was reconstituted at 10 µg/mL in sterile 4 mmol/L HCl containing 0.1% BSA. Aliquots were kept at −80°C and discarded after 3 months. Galunisertib was provided by Eli Lilly and Company. For in vitro experiments, galunisertib was freshly suspended in a formulated vehicle (1% carboxymethylcellulose, 0.5% SDS, 0.085% povidone, and 0.05% antifoam Y-30 emulsion) and kept at 4°C for up to 1 week. Galunisertib was dissolved in DMSO at 10 mmol/L and kept at −20°C as a stock solution for in vivo experiments.

Gene expression analysis

Affymetrix Human Exon Array data (manuscript in preparation; see https://ocg.cancer.gov/programs/target/research) of 249 primary neuroblastoma tumor specimens obtained at diagnosis was normalized by quartile normalization and summarized using robust multichip average (Affymetrix Power Tools software package version 1.12). This dataset includes samples from 219 patients with high-risk (68 with amplified and 151 with nonamplified MYCN) and 30 with low-risk primary tumors. The transcript level data of core probe sets for each sample were averaged on the basis of gene symbol annotations provided by the manufacturer (17,422 unique genes). To identify relative expression of genes in neuroblastomas, the percentile values of TGFBR1, TGFBR1, TGFBR2, TGFBR2, TBX21, IFNG, NTRK1, and MYCN were computed from the cumulative distribution function for each sample’s gene profile. As an independent dataset, Agilent single-color expression profiles of 478 samples were downloaded from the GEO GSE16716 dataset. Patients with stage 4S disease in this latter dataset (n = 62) were excluded from analysis to allow comparison with our Human Exon Array data. Expression profiles from the resulting cohort of 416 tumors from patients with high-risk (n = 135), intermediate-risk (n = 34), or low-risk (n = 247) neuroblastoma were used to assess expression of TGFBR1, TGFBR1, TGFBR2, TBX21, IFNG, as well as NTRK1 and MYCN as internal controls. Concordant results were obtained between our neuroblastoma dataset and the GEO GSE16716 dataset.

Neuroblastoma patient plasma SMAD activity assay

The Cignal Lenti SMAD Reporter, purchased from SA Biosciences, is comprised of lentiviral particles containing the firefly
gene under the control of SMAD transcription response elements. CHLA-255 cells expressing a high level of Renilla luciferase (CHLA-255-hRL) were transduced by this SMAD reporter lentivirus, and the stable CHLA255-hRL-SmadFluc cell line was established by puromycin selection. To validate detection of SMAD activity, 5 × 10^4 CHLA255-hRL-SmadFluc cells were seeded into each well of a 96-well plate overnight and then cultured with various doses of galunisertib with and without recombinant human TGFβ1 (10 ng/mL). Beetle luciferin (7 µL, 5 mg/mL; Promega) was added to each well for 5 minutes in the dark. Activation of SMAD transcription response elements by TGFβ1 or patient plasma resulted in expression of firefly luciferase, which when activated by luciferin, was measured as luminescence using the GloMax Multi-Detection System (Promega; model #E8032).

To determine whether neuroblastoma patient blood or bone marrow plasma could activate SMAD2, samples from 17 patients with high-risk neuroblastoma (1:10 dilution with 1% FBS-IMDM) were added into wells with or without galunisertib for 18 or 36 hours.

Western blotting for pSMAD2
Cell pellets were lysed with 1 × RIPA buffer including 1 × Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Proteins (40 µg) were analyzed on a Novex 4% to 20% Tris-Glycine gel (Life Technologies) using Novex Tris-Glycine SDS Running Buffer (Invitrogen/Thermo Fisher Scientific). Proteins were transferred to a pure nitrocellulose membrane (Bio-Rad) in Novex Tris-Glycine Transfer Buffer (Invitrogen/Thermo Fisher Scientific) plus 20% methanol. The following antibodies were used: rabbit polyclonal anti–phospho-SMAD2 (Ser465/467) antibody (Cell Signaling Technology), rabbit polyclonal anti-SMAD2/3 antibody (Cell Signaling Technology), and rabbit polyclonal anti–β-actin (N-21) antibody (Santa Cruz Biotechnology, Inc.). Antibody-reactive proteins were detected with HRP-labeled goat anti-rabbit IgG (Bio-Rad) and ECL substrate (Denville Scientific). NewBlot Nitro Stripping Buffer (Li-Cor) was used to strip the anti–phospho-SMAD2 antibody before addition of antibodies for detection of other proteins.

Immunohistochemical staining of phospho-SMAD2 in human neuroblastoma tumors formed in NSG mice
Tumor tissues from kidneys of NSG mice in different treatment groups were placed formalin (Fisher Scientific Company LLC) for 2 days and then into 75% ethanol at 4°C. Formalin-fixed, paraffin-embedded sections in Leica BOND-MAX (Leica Microsystems) were heated for 30 minutes in Bond Epitope Retrieval Solution 2 (No. AR9640; Leica Biosystems Newcastle Ltd.). Sections then were incubated for 2 hours at room temperature with anti–phospho-SMAD2 antibody (Ser465/467; rabbit polyclonal antibody; EMD Millipore) at a dilution of 1:500, followed by a poly-horseradish peroxidase–conjugated goat anti-rabbit antibody (Leica). Kidney tissue of a normal NSG mouse was stained as a negative control.

Flow cytometry
Cell surface staining was performed, as previously described (24, 25). Briefly, cells were washed twice in FACS buffer (PBS with 0.1% NaN3 and 0.1% BSA) and centrifuged for 10 minutes at 400 × g. Antibodies listed in Supplementary Table S1 were added in the dark at 4°C using concentrations previously determined by titration. Isotype-matched irrelevant mAbs were used to define nonspecific staining. Cells were incubated at 4°C for 90 minutes and washed twice in FACS buffer. Dead cells were excluded according to positivity for DAPI. Cell aggregates were excluded from analysis by gating out events exhibiting high forward and side light scatter. Flow cytometry analysis was performed using a BD LSR II flow cytometer with DIVA software (BD Biosciences) and FCS Express software (DeNovo Software). The Stain Index was calculated using values of median fluorescence intensity (MFI) and robust SD (rSD) as follows: (MFI of viable aNK cells stained with specific antibody – MFI of viable aNK cells stained with an isotype-matched irrelevant antibody)/(2 × rSD of the isotype control).

**NK cytotoxicity assay**
Frozen K562.mblb21-expanded aNK cells (24) were cultured in 10% FBS–RPMI1640 with 100 U/mL of IL2 for 24 hours and then were pretreated in individual wells of 6-well plates for 48 hours with (i) 5 µmol/L galunisertib, (ii) 10 ng/mL human TGFβ1, (iii) galunisertib for 30 minutes followed by TGFβ1, or (iv) 24 hours with TGFβ1 followed by treatment with galunisertib for an additional 24 hours. For the NK cytotoxicity assay, neuroblastoma cell lines (CHLA-255-Fluc and CHLA-136-Fluc) were labeled with calcine-AM for 30 minutes and washed once (26). A total of 1 × 10^4-labeled CHLA-255-Fluc or CHLA-136-Fluc were seeded into individual wells of a 96-well plate, and 5 × 10^4 aNK cells pretreated as described above were added to neuroblastoma cells at a ratio of 1:2. Dinutuximab was added to the indicated wells at a concentration of 1 µg/mL for the ADCC assay. The plate was incubated at 37°C in 5% CO2 for 6 hours, and surviving tumor cells were quantified as calcine-containing cells using a digital imaging microscopy system (DimScan; ref. 26).

**Luminex assay**
Granzyme A and perforin were measured using a custom-plex bead array from EMD Millipore following the manufacturer's instructions with a Luminex-200 instrument (Luminex Corporation), as described previously (24).

**Intrarenal neuroblastoma model and treatment of NSG mice**
All in vivo experiments utilized 4- to 6-week-old male and female NSG (NOD.Cg-Pkbk<sup>−/−</sup>IL2rg<sup>−/−</sup>/SzJ) mice, which were bred in-house, genotyped for colony maintenance, and housed in a pathogen-free environment. Mice were implanted using our previously described intrarenal xenograft model of neuroblastoma (27). Briefly, 1 × 10<sup>6</sup> cells from neuroblastoma cell lines or PDX cells in 100-µL PBS were injected in the left kidney of mice. Tumor growth in mice injected with luciferase-expressing cell lines was assessed by bioluminescence imaging using a Xenogen IVIS 100 instrument (IVIS Lumina XR System; Caliper Life Sciences). All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Children’s Hospital Los Angeles.

Mice in groups receiving aNK cells and dinutuximab were intravenously injected with 1 × 10<sup>6</sup> aNK cells (immediately after thawing) plus 15 µg mAb twice per week per mouse for 4 weeks, as described previously (24). IL2 (2 µg/mouse) and IL15 (4.9 µg/mouse) were injected intraperitoneally at the same time as dinutuximab and aNK cells. Mice in galunisertib treatment groups were gavaged twice a day with 75 mg/kg of galunisertib (16) suspended in formulated vehicle, as described above. Mice not receiving galunisertib were gavaged twice a day with the formulated vehicle solution alone.
Statistical analysis

Data were analyzed using Stata statistical software (version 11.2) and are represented as means ± standard deviation (SD) unless otherwise stated. ANOVA was performed to determine the significance of observed differences. Analysis of tumor bioluminescence data transformed the photon flux for each mouse using the log (flux + 1) transformation and then area under the growth curve (AUC) was calculated. The AUC values were used in the analysis to compare differences in tumor photon flux between treatment groups. Mouse survival time was defined as the length of time (in days) from the tumor injection date until the end of the study or time of sacrifice due to disease progression. Censored normal regression was used to examine whether any difference in survival time existed due to treatments. The censored Wilcoxon test was used to examine the difference in the survival curves among the different treatment groups. A P value of <0.05 was considered statistically significant.

Results

**TGFBR1 and TGFBR2** genes are expressed at high-risk human neuroblastoma tumors, and TGFβ activity is present in blood and bone marrow plasma from neuroblastoma patients

Exon gene expression in 249 primary neuroblastoma tumors was analyzed using Affymetrix human exon arrays (17,422 genes). TGFBR1 and TGFBR2 genes were expressed at high levels relative to all genes analyzed, and the median expression of TGFBR1 was above the 30th percentile (Fig. 1). MYCN expression was at the 100th percentile in the MYCN-amplified group of tumors, and NTRK1 expression was high in the low-risk group, as expected. mRNA expression was at less than the 5th percentile for IFNγ (IFNG) and above the 30th percentile for its controlling transcriptional regulator TBX21 (the human ortholog of the mouse Tbet/Tbx21 gene). Median percentile expression of TGFBR1 and TGFBR2 and high percentile expression of their receptors with low expression of IFNG suggest activity of the TGFβ signaling pathway in neuroblastomas.

Next, we used the neuroblastoma SMAD reporter cell line CHLA255hRL-SmadFluc to determine whether blood and bone marrow plasmas from neuroblastoma patients activate the SMAD pathway and whether this activation could be suppressed by the TGFBR1 inhibitor galunisertib. First, we performed serial 5-fold dilutions of galunisertib and found that a clinically achievable dilution of galunisertib (5 μmol/L) gave maximum inhibition of SMAD activity induced by 10 ng/mL of TGFβ1 without cell toxicity (Fig. 1B). Then, using 5 μmol/L galunisertib and the CHLA255hRL-SmadFluc reporter cell line, we observed that SMAD activity was induced by blood and bone marrow plasma obtained from 17 neuroblastoma patients (Fig. 1C) and that this activity could be inhibited by galunisertib. These results suggest that TGFβ1 and/or TGFβ2 are present in blood and bone marrow from neuroblastoma patients.

Galunisertib decreases suppression of aNK cells by TGFβ1

Having established that galunisertib reduces SMAD activity in neuroblastoma cells, we examined whether it might reduce the effects of TGFβ1 on aNK cells. Ex vivo–propagated aNK cells were cultured with galunisertib (5 μmol/L), TGFβ1 (15 ng/mL), or the combination of galunisertib with TGFβ1 for 18 hours, and lysates were subjected to immunoblot assay to detect phospho-SMAD2, total SMAD2, and β-actin. Figure 2A shows strong induction of
SMAD2 phosphorylation in aNK cells by TGFβ1 and strong inhibition of this phosphorylation by galunisertib. TGFβ1 also decreased aNK cell expression of the cytotoxicity receptors DNAM-1, NKp30, and NKG2D (Fig. 2B), as reported by others for human NK cells cultured for 2 to 7 days with IL2 or IL15 (3, 29). TGFβ1 also decreased aNK cell expression of the membrane-bound form of the death ligand TRAIL (Fig. 2B), which we have previously shown to supplement the cytotoxicity of aNK cells against neuroblastoma cells (30). Our experiments did not demonstrate that TGFβ1 affected expression of NKp46, NKG2A,
NGK2C, or CD16 (data not shown). The approximately 3-fold decrease in the Stain Index for DNAM-1, NKp30, NGK2D, and TRAIL induced by TGFβ1 was inhibited by galunisertib when added either 30 minutes before or 24 hours after commencing incubation with TGFβ1 (Fig. 2B). Among the receptors for TRAIL, TRAIL receptor 2 (TRAIL-R2) was expressed by the neuroblastoma cell lines CHLA-255-Fluc and CHLA-136-Fluc and by PDX cells COG-N-415x (Fig. 2C). There was little or no expression of TRAIL-R1, TRAIL-R3, or TRAIL-R4 (data not shown). Ligands for DNAM-1 (CD112 and CD155) were also expressed by CHLA-255-Fluc and CHLA-136-Fluc cells and by PDX cells COG-N-415x (Fig. 2C). The ligand for NKp30 (B7-H6) was expressed by the cell lines, but not the PDX cells. Among ligands for NGK2D (MICA, MICB, ULBP1, ULBP2/5/6, ULBP3), CHLA-255-Fluc cells demonstrated clear expression of only ULBP3, CHLA-136-Fluc cells expressed little or none of the ligands, and COG-N-415x cells expressed low levels of MICA and ULBP2/5/6 with minimal expression of the other three ligands. Importantly, neither TGFβ1 nor galunisertib affected the expression of these ligands or of GD2 on the neuroblastoma cell lines in vitro (Supplementary Fig. S1).

Because perforin and granzymes are important for NK cell cytotoxicity (31, 32), we next examined the effect of TGFβ1 and galunisertib on aNK cell release of perforin and granzyme A. As expected, TGFβ1 (10 ng/mL) reduced, by 30% or more (P < 0.02), the release of both perforin and granzyme A from aNK cells cultured alone or from aNK cells cultured in direct contact with neuroblastoma cells (Fig. 2D). TGFβ1-induced inhibition of perforin and granzyme A secretion was prevented by 5 μmol/L of galunisertib (Fig. 2D; P < 0.05). Intracellular immunostaining for perforin and granzyme A expression indicated no effect of TGFβ1 or of galunisertib (Supplementary Fig. S2), suggesting that TGFβ1 inhibits the release of perforin and granzyme A from aNK cells rather than inhibiting their intracellular expression. Taken together, these results indicate that galunisertib inhibits multiple suppressive effects of TGFβ1 on the cytotoxic mechanisms of aNK cells.

Having established that galunisertib significantly inhibits effects of TGFβ1 on aNK cells, we examined whether it also prevents inhibition of their cytotoxicity by TGFβ1. aNK cells that have been propagated using K562 mblL21 cells are known to be highly cytotoxic, and this cytotoxicity against multidrug-sensitive and -resistant neuroblastoma cell lines in vitro is increased by dinutuximab (24). We found that aNK cytotoxicity against CHLA-255-Fluc (moderately multidrug sensitive) and CHLA-136-Fluc neuroblastoma cells (multiresistant resistant; ref. 33) was inhibited by TGFβ1, but this inhibitory effect was significantly reversed by addition of 5 μmol/L galunisertib 30 minutes before (Fig. 3). Importantly, addition of galunisertib 24 hours after TGFβ1 also inhibited the reduction in aNK cytotoxicity by TGFβ1. These findings demonstrate that galunisertib inhibits the suppressive effects of TGFβ1 on aNK cell–mediated direct cytotoxicity and ADCC against neuroblastoma cells.

Galunisertib decreases phosphorylation of SMAD2 in neuroblastoma xenografts in NSG mice

To evaluate the inhibitory effect of galunisertib on the TGFβ pathway in neuroblastoma tumors, intrarenal tumors were examined for phospho-SMAD2 on day 36, 21, or 27 from mice injected in their left kidneys with CHLA-255-Fluc, CHLA-136-Fluc, and patient-derived xenograft COG-N-415x cells, respectively. These mice were injected intravenously twice a week with dinutuximab mixed with K562 mblL21-propagated aNK cells, starting three days after intrarenal injection of neuroblastoma cells. IHC demonstrated phospho-SMAD2 in nuclei of tumors that were untreated or treated with dinutuximab and aNK cells (Fig. 4A–C, brown color). Compared with untreated tumors, phospho-SMAD2 was decreased in neuroblastomas treated with galunisertib and was decreased the greatest in tumors treated with the combination of galunisertib, dinutuximab, and aNK cells. These results indicate that galunisertib is able to penetrate human neuroblastoma xenografts where it reduces SMAD2 pathway activation.

Galunisertib enhances anti-neuroblastoma activity of dinutuximab plus human aNK cells in NSG mice

The ability of galunisertib to inhibit growth of human neuroblastoma tumors and improve survival of NSG mice treated with dinutuximab plus human aNK cells was tested in mice bearing tumors formed by intrarenal injection of CHLA-255-Fluc, CHLA-136-Fluc, and COG-N-415x cells. K562 mblL21-propagated aNK cells were mixed with dinutuximab and injected intravenously twice weekly, starting 3 days after intrarenal injection of neuroblastoma cells. Bioluminescence imaging was performed for mice injected with luciferase-expressing CHLA-255-Fluc or CHLA-136-Fluc cells, and decreases in bioluminescence were observed in mice treated with the combination of dinutuximab, aNK cells, and galunisertib (Fig. 5A). In contrast, galunisertib alone had no...
dinutuximab in 0.41). Importantly, the value of +dinutuximab, B, + aNK 10 in vitro MYCN Tumors from COG-N-415x expression both in high-risk tumors TGF[b] and TGF[a] lus with TGFBR1, TGFBR2 = + aNK 0.003 and 0.02 for TGFBR1, = 0.08), and treatment with galunisertib alone did not significantly decrease tumor growth (P = 0.41). Importantly, the combination of galunisertib with dinutuximab and aNK cells significantly extended survival of mice injected with CHLA-255-Fluc, CHLA-136-Fluc, or COG-N-415x neuroblastoma cells compared with either untreated mice or mice treated with dinutuximab and NK cells (Fig. 5B–D). Taken together, these data indicate that the addition of galunisertib significantly enhances the antitumor effect of dinutuximab and aNK cells in NSG mice implanted with neuroblastoma cell lines or a PDX.

Discussion

Using microarray gene expression profiling of 249 untreated primary neuroblastomas from patients, we show TGFBR1, TGFBR2, TGF[b], and TGF[b] expression both in high-risk tumors that have either amplified or nonamplified MYCN and in low-risk neuroblastomas. We also show very low expression of IFNG in these tumors, consistent with TGF[b]-mediated suppression of NK cells (13). A previous study of 61 neuroblastomas of all clinical stages using conventional RT-PCR and electrophoresis also showed expression of TGFBR1, TGFBR2, and TGF[b] (34). In addition, we show for the first time that bone marrow and blood plasmas from patients induce SMAD signaling in a reporter neuroblastoma cell line and that galunisertib, a small-molecule inhibitor of the TGF[b] signaling pathway that is in phase I and II clinical trials (15), blocks this activity. In agreement with these findings, we show that phospho-SMAD2, which accumulates in cell nuclei downstream of TGF[b] signaling (35), can be detected in untreated neuroblastomas growing in NSG mice. Treatment of mice with galunisertib inhibited phosphorylation of SMAD2 in these tumors, which indicates its activity in the tumor microenvironment.

We and others have previously demonstrated that aNK cells can be propagated using K562.mblL1 cells to numbers that should be sufficient for adoptive cell therapy in humans (22–24). Here, we demonstrate for the first time that TGF[b] inhibits both direct killing and ADCC of neuroblastoma cells by such aNK cells in vitro and that galunisertib can substantially reverse this inhibition. Restoration of ADCC by galunisertib did not involve modulation of CD16 expression. Instead, our findings show that galunisertib treatment of aNK cells in vitro inhibits TGF[b] induced SMAD2 phosphorylation and significantly restores expression of DNAM-1, NKP30,
NKG2D, and TRAIL and release of perforin and granzyme A, which could contribute to the observed reversal of TGFβ1-mediated inhibition of cytotoxicity. The ability of a specific TGFBR1 inhibitor to inhibit SMAβD2 phosphorylation, an indicator of TGFBR1 activity, and to reverse multiple downstream effects of TGFβ1 suggests that TGFBR1-mediated signals are key regulators of multiple components of the cytotoxicity of aNK cells and that this regulation may be reversible upon specific treatment.

Importantly, addition of galunisertib to dinutuximab and aNK not only prevented in vivo phosphorylation of SMAD2 but enhanced the immunotherapy and survival of NSG mice bearing CHLA-255-Fluc or CHLA-136-Fluc cells. One mouse injected with CHLA-136-Fluc cells in the untreated group exhibited a weak signal but had a large tumor when sacrificed on day 49. Mouse X1 died on day 16 during anesthesia for imaging; mouse X2 died on day 21 from unknown reasons, with no observable tumor.

Figure 5. Effect of galunisertib on growth of neuroblastoma tumors and survival of NSG mice. Mice were treated as in Fig. 4. A, Bioluminescence imaging on day 23 for mice bearing CHLA-255-Fluc or CHLA-136-Fluc cells. One mouse injected with CHLA-136-Fluc cells in the untreated group exhibited a weak signal but had a large tumor when sacrificed on day 49. Mouse X1 died on day 16 during anesthesia for imaging; mouse X2 died on day 21 from unknown reasons, with no observable tumor. B, Kaplan-Meier survival plot for mice injected with CHLA-255-Fluc cells. P values comparing treatment groups are shown in the inserted box. C, Kaplan-Meier survival plot for mice injected with CHLA-136-Fluc cells. D, Kaplan-Meier survival plot for mice injected with PDX COG-N-415x cells. Dinutux, dinutuximab.

In preclinical studies of cancer, galunisertib has been reported to enhance the activity of paclitaxel or sorafenib but to have limited activity by itself (17, 18). Treatment with galunisertib alone inhibited the growth of only 2 of 13 lung and prostate carcinoma cell lines grown in nude mice (39) and had little or no effect against triple-negative breast cancer xenografts, even though pSMAD2 was decreased (17). Galunisertib enhanced paclitaxel treatment of breast cancer stem cells by blocking paclitaxel-induced interleukin-8 transcription and associated cell proliferation, and treatment of breast cancer xenografts with both drugs prevented reestablishment of tumors (17). Galunisertib decreased phosphorylation of SMAD2 in hepatocellular carcinoma cells, limiting their invasive properties ex vivo and potentiating sorafenib-mediated apoptosis and a decrease in proliferation in vitro (18). Our...
study, which did not show galunisertib alone to be active against neuroblastomas growing in NSG mice, is in agreement with these previous reports. Taken together, these findings indicate that combining galunisertib with other therapeutic modalities is necessary for realizing its anticancer potential.

Our study is the first to show that galunisertib can act as a combinatorial agent with immunotherapy, enhancing mAb and NK cell–based treatment of human tumor xenografts. We show that galunisertib administered to NSG mice according to a previously established pharmacokinetic/pharmacodynamic protocol (16) enhances the anti-neuroblastoma effect of dinutuximab with adoptively transferred aNK cells. These findings provide preclinical support for testing galunisertib in combination with dinutuximab in clinical trials for neuroblastoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: H.C. Tran, Z. Wan, S. Aghazadeh, R.C. Seeger

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