Hedgehog Pathway as the Target in Rhabdomyosarcoma with Gli1 Gene Amplification

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INTRODUCTION: Hedgehog (Hh) pathway inhibition is being actively investigated in clinical trials for metastatic colorectal cancer, ovarian cancer, medulloblastoma and other solid tumors. One such inhibitor, GDC-0449 (Vismodegib), has shown positive results in phase II clinical trial on advanced basal cell carcinoma. Response to Hh pathway inhibition has been shown for both GDC-0449 and GANT61 for in vitro, non-Gli gene amplified RMS models. Our interest lies in the potential use of Hh pathway inhibition for rhabdomyosarcoma, in which Gli1 gene amplification has been reported in ~25% of human cases [1]. The Objective of this study was to evaluate the effects of hedgehog (Hh) pathway inhibition at two different levels in the alveolar rhabdomyosarcoma (ARMS) cell line RMS-13 harboring Gli1 gene amplification. Pathway inhibition was conducted at the level of Smoothened (Smo) with the drug GDC-0449 and downstream at the target Gli with GANT61. Our Hypothesis was that in this Gli-amplified model, Hh inhibition would only be possible at the downstream target Gli, not at Smo.

METHODS: For viability experiment, human ARMS cell line RMS-13 was seeded in 24-well plates and exposed to GDC-0449 or GANT61 for 1 to 4 days. Next, cell viability was measured using colorimetric MTT assay. Viability in treated wells was normalized to the viability of untreated controls. Experiment was conducted 4 times with consistent results. For the Hh pathway activity experiment, cells were seeded in 6-well plates and treated with 5 uM of each drug individually for 48 hours. Next, RNA was extracted and RT-qPCR performed. Expression levels were calculated using ΔΔCt method with REST® software. Experiment was repeated 3 times with consistent results. For the visualization of fragmented DNA, cells were seeded in 6-well plates and exposed to GANT61 for 1-3 days. After 1 day exposure, both suspended and adherent cells were collected. After 2 and 3 day exposure, all cells were in suspension. Fragmented DNA was extracted using Suicide Track™ DNA Ladder Isolation Kit (Calbiochem) according to manufacturer’s protocol. For clonogenic assay, cells were seeded in 6-well plates at low densities. After 8 hours attachment, 1uM of GDC-0449 or GANT61 were added. After further 8 day incubation, cells were fixed and colonies stained with crystal violet. Colonies were counted and surviving fraction calculated.

RESULTS: From viability experiments, RMS-13 cell lines showed marked decrease in viability after Gli protein inhibition by GANT61 at 10 or 30 uM. In contrast, RMS-13 cells were resistant to GDC-0449 treatment even after prolonged treatment at both 10 and 30 uM doses (Fig. 1). Gli1 being the transcription factor, it activates transcription of its own gene, PTCH1 (Hh receptor) and other genes involved in proliferation. To analyze Hh pathway activity after treatments, we measured transcripts of Gli1 and Pch1 with RT-qPCR. We saw 4-fold (p<0.001) and 7-fold (p<0.001) decrease in expression of Gli1 and Pch1 genes, respectively, with GANT61 treatment (Fig. 2) but not with GDC-0449 treatment (data not shown).

To better understand what was causing the decrease of overall viability after GANT61 exposure, we extracted fragmented DNA, which showed a smear of randomly cleaved DNA, a pattern characteristic to necrotic cell death (Fig. 3) rather than the characteristic “ladder pattern” seen with apoptosis. It is known that hedgehog pathway regulates expression of genes involved in cell cycle regulation and proliferation. We conducted colony formation assay to see if down-regulation of these genes had a role in the overall effect of decreased viability in RMS-13 cells. Analysis showed decrease in the number of cells capable of proliferation upon subtoxic (1uM) GANT61 exposure (Fig. 4). No such significant decrease was seen after GDC-0449 exposure.

DISCUSSION: In this study, we demonstrated response to GANT61 and failure of response to GDC-0449 treatment. This can be explained by the fact that RMS-13 harbors 30-fold Gli1 gene amplification, which is downstream of Smo. Target of GDC-0449. Decrease in viability was partially due to necrotic cell death. GANT61 has been shown to induce apoptosis in leukemic cells, but at least with DNA fragmentation assay this was not true in the RMS cell line model. Although other more specific experiments will be conducted to explore apoptosis as the mechanism of GANT61 induced cell death, GANT61 also demonstrated ability to decrease proliferative capacity of the RMS-13 cell line by its influence on the cell cycle machinery. In conclusion, these data suggest that Smo cannot be used as the target in RMS cases with Gli amplification.

SIGNIFICANCE: Current treatment protocols for rhabdomyosarcoma generally include surgical excision with wide margins, pre- and post-operative chemotherapy, and adjunctive radiation delivered prior to surgery, each carrying undesirable side effects. More specifically targeted therapies would potentially improve outcome and lessen adverse effects.

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REFERENCES