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α 5-GABAA receptors negatively regulate MYC-amplified medulloblastoma growth

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Abstract

Neural tumors often express neurotransmitter receptors as markers of their developmental lineage. Although these receptors have been well characterized in electrophysiological, developmental and pharmacological settings, their importance in the maintenance and progression of brain tumors, and importantly, the effect of their targeting in brain cancers remains obscure. Here, we demonstrate high levels of *GABR5*, which encodes the α -subunit of the GABAA receptor complex, in aggressive MYC-driven, “Group 3” medulloblastomas. We hypothesized that modulation of α -GABAA receptors alters medulloblastoma cell survival and monitored biological

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Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' contributions

Project conceived by YJC and experimental plan developed and directed by YJC, SSG, SDW, FEJ, SLP. Manuscript written by YJC, with contributions from SSG and SDW. SSG, SDW, NT, VA, JPF, BN, YT, FY, TCA performed maintenance of medulloblastoma cells lines and performed all molecular biology experiments (qPCR, cell viability assays, transfections, cell line irradiation, Western blot). SR and JC synthesized and provided QHii066. RJR synthesized recombinant DBI for use in experiments. HS developed and performed all electrophysiology experiments. BK, SSG and ML performed immunofluorescence studies on mouse xenograft and primary human FFPE tissue. JP, DM, PT and SSG performed mouse studies.

and electrophysiological responses of GABR5-expressing medulloblastoma cells upon pharmacological targeting of the GABAA receptor. While antagonists, inverse agonists and non-specific positive allosteric modulators had limited effects on medulloblastoma cells, a highly specific and potent $\alpha 5$ -GABAA receptor agonist, QHii066, resulted in marked membrane depolarization and a significant decrease in cell survival. This effect was GABR5 dependent and mediated through the induction of apoptosis as well as accumulation of cells in S and G2 phases of the cell cycle. Chemical genomic profiling of QHii066-treated medulloblastoma cells confirmed inhibition of MYC-related transcriptional activity and revealed an enrichment of HOX5 target gene expression. siRNA-mediated knockdown of HOX5 markedly blunted the response of medulloblastoma cells to QHii066. Furthermore, QHii066 sensitized GABR5 positive medulloblastoma cells to radiation and chemotherapy consistent with the role of HOX5 in directly regulating p53 expression and inducing apoptosis. Thus, our results provide novel insights into the synthetic lethal nature of $\alpha 5$ -GABAA receptor activation in MYC-driven/Group 3 medulloblastomas and propose its targeting as a novel strategy for the management of this highly aggressive tumor.

Keywords

medulloblastoma; GABRA5; QHii066; HOXA5; MYC

Introduction

Medulloblastomas are the most common malignant brain tumors in children and a significant cause of childhood cancer-related deaths [9]. Recent large-scale genomic studies have detailed their genetic heterogeneity and have identified four broad molecular subgroups within this disease: SHH, WNT, Group 3, and Group 4 [8,19,22,34]. Importantly, these studies have revealed children with Group 3 medulloblastomas have a significantly worse prognosis relative to patients with other medulloblastoma subtypes [8,19,33]. Group 3 medulloblastomas are characterized by oncogenic MYC signaling, often through high-level MYC-amplification, and are particularly resistant to conventional cancer treatments such as radiation and chemotherapy, even at maximally tolerated doses [7,26]. Unfortunately, attempts at developing drugs that directly target the MYC protein have been met with only limited success. Therefore, efforts have shifted towards identifying alternate biological pathways or genetic factors (“synthetic lethals”) that, when targeted, are able to shut down oncogenic MYC-signals.

We previously reported the enrichment of genes associated with GABA pathway signaling in MYC-driven/Group 3 medulloblastomas, largely from increased *GABR5* expression [8]. *GABR5* encodes the α subunit of the GABAA receptor complex, a pentameric structure composed of two α , two β and one γ subunit. GABAA receptors function primarily as ligand-gated chloride channels, which bind to GABA, other endogenous peptides and a host of pharmacological agents at defined sites around/within the receptor complex [20]. Binding specificity is mediated in part by the existence of multiple $\alpha(1-6)$, $\beta(1-3)$ and $\gamma(1-2)$ subunits, which are also temporally and spatially dynamic. The most ubiquitous and abundant GABAA receptor complexes in the central nervous system contain $\alpha 1$ subunits [32], while α subunit-containing GABAA receptors are more restricted in their expression with the highest levels noted in distinct sets of neurons in the hippocampus, cerebellum and sensory-related brain regions [28].

Despite the multiple combinations of receptor subunits and their varied temporospatial expression, the end result of GABAA receptor activation (by ligand or chemicals) is Cl^- flux across the cell membrane and subsequent perturbation of cell membrane potential.

Alteration of the cell membrane potential is followed by a series of second messenger events, most often mediated by mobilization of Ca^{++} and its related signaling cascades. From a functional standpoint, GABAA receptor activation typically results in inhibitory neurotransmission except in prenatal and early postnatal development where GABAA receptor signaling is excitatory due to distinct, age-dependent differences in intracellular chloride levels in developing neurons (increased) compared to mature neurons [10].

Evidence also supports the role of GABA pathway signaling as a critical regulator of stem cell maintenance by limiting transition of cells through the G2 cell cycle checkpoint in a PI3K and γ -H2AX-dependent manner [3]. GABA signaling has been shown to control both embryonic stem cell and peripheral neural crest cell proliferation, blunting rapid proliferation in favor of a more tempered rate of proliferation, ensuring genome integrity and restricting overall stem cell number and the size of the neural stem cell niche [3,12]. GABA-induced depolarization in cortical progenitors and neuronal precursor cells has also been shown to inhibit DNA synthesis and cell cycle progression, respectively, through activation of voltage-dependent Ca^{++} channels [17].

From a pharmacological standpoint, an abundance of small molecules have been developed that modulate GABAA receptor activity. These compounds include several FDA-approved drugs that are used clinically as anxiolytics, anti-seizure and anesthetics based on their potentiation of GABA-mediated inhibitory neurotransmitter activity. An arsenal of tool compounds has also been generated that target specific subunits of the GABAA receptor, including pure agonists, inverse agonists, antagonists and allosteric modulators (positive and negative), many of which are being optimized for clinical use in neuropsychiatric disease [40].

Given the growing evidence for GABA pathway regulation of stem and neural stem cell proliferation, the current arsenal of pharmacological reagents available to modulate GABAA receptor activity and the identification of high levels of GABR5 expression in medulloblastoma, we sought to clarify the role of $\alpha 5$ -GABAA receptor signaling in medulloblastoma and investigated whether targeting this pathway in Group 3/MYC-driven medulloblastomas could affect tumor cell survival.

Materials and Methods

Ethics statement

Ethics approval was granted by the relevant human IRB and/or animal ethics IACUC research committees of Stanford University and Boston Children's Hospital.

Medulloblastoma cell lines

The medulloblastoma cell lines D425, D283 and D556 were a kind gift from Dr. Darrell Bigner. MB002 cells were derived from an autopsy specimen of the leptomeningeal compartment from a child with metastatic, treatment-refractory (chemotherapy only) medulloblastoma. The MB002 primary tumor displayed histological features of large cell medulloblastoma and evidence for *MYC*-amplification and gene expression markers consistent with Group 3 medulloblastoma (Bandopadhyay et al., *manuscript in preparation*). DAOY cells were obtained from the American Tissue Culture Collection.

Gene expression and bioinformatics analysis

Evaluation of GABRA5 gene expression in primary medulloblastomas was performed using the R2 microarray analysis and visualization platform (<http://r2.aml.nl>) and previously published datasets [8, 19, 25].

Gene expression data was generated from RNA isolated from D425, D283, D556, MB002 and DAOY medulloblastoma cells (treated with QH-ii-066 and DMSO as a control) using Illumina HT12 Bead Arrays. Prior to microarray analysis, RNA quality was assessed on a Bioanalyzer (ABI) and RINs were greater than 9 for all samples analyzed. GCT files were then generated from IDAT files using the GenePattern software suite (<http://www.broadinstitute.org/cancer/software/genepattern>). Data was further normalized using rank-invariant set normalization (as described in detail in [37]).

Gene set enrichment analysis (GSEA) was performed as previously described (<http://www.broadinstitute.org/software/gsea>) [31] using the “Canonical Pathways (CP)” and “Chemical and Genetic Perturbation (CGP)” gene sets in MSigDB (<http://www.broad.mit.edu/gsea/msigdb>).

RNA Extraction

RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentration was measured using a NanoDrop Spectrophotometer (NanoDrop Technologies).

Real-time quantitative reverse transcription-PCR (qPCR)

Gene Expression assays for *GABRA5*, *B2M*, *HOXA5* and *p53* were purchased from Applied Biosystems. Analysis was carried out using the Applied Biosystems 7300 q-RT-PCR System. The gene expression delta cycle threshold (Δ CT) values of mRNAs in each sample were calculated by normalizing with the internal control B2M, and relative quantification values were plotted. All reactions were done in triplicate, and at least 3 independent experiments were performed to generate each data set.

Cell Viability Assays: MTS Assay

Suspended cell-lines were seeded at 20 000 cells per well and adherent cell lines were suspended at 2500 cells per well in 96-well plates (Corning) in 75 μ L of an antibiotic-free medium. Each sample was plated in pentuplicate. QH-ii-066 (synthesized by James Cook's laboratory) and/or *cis*-diamineplatinum (II) dichloride (cisplatin; Sigma) was added 48 hours post-plating at 4 $\times$ concentrations made up to 25 μ L in an antibiotic-free medium.

Other compounds used in a similar manner include the following: GABA, phenobarbitone, diazepam, picrotoxin, etoposide, bumetanide, PWZ029 (moderate inverse agonist of GABA), XLI093 (*GABRA5* antagonist), SH053-F-RCH3 (*GABRA5* agonist) and Mibefradil.

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay with CellTiter 96 AQueous One Solution Reagent (Promega) following the manufacturer's protocol. After incubating at 37°C for 1 hour, the optical density of each well was measured with a spectrophotometric microplate reader (Bio-Rad) at 490 nm.

Soft agar colony formation assay

Anchorage-independent colony formation was assayed by a soft-agar assay. Briefly, 1X10³ D425 or D556 medulloblastoma cells (treated with QHii066 or DMSO) was seeded as a single cell suspension in 0.4% SeaPlaque Agarose in DMSO (Lonza) which was then overlaid on a pre-prepared base layer of 1% agarose in 6 well plates. Plates were then incubated in a humidified 37°C incubator with 5% CO₂ for 14 days and were subsequently stained with 1 mL of 0.01% crystal violet (Sigma) in 1X PBS for 30 minutes. Colonies were visualized and captured by a Nikon Eclipse TE 2000-S inverted microscope and counted in

5 random fields for all the conditions tested. Bar graphs were generated by combining data from three independent experiments with each condition plated at least in duplicate.

Lentiviral production and transduction

Mission shRNAs against GABRA5, HOXA5 and p53 (5 different shRNA constructs against each gene) were purchased from Sigma. Each construct was transfected into 293T cells and pseudovirus particles were generated according to manufacturer's instructions. Subsequently, D425 target cells were infected at a MOI of 2 in the presence of 5 µg/ml Polybrene (Sigma). Forty-eight hours after viral infection selective pressure was exerted on transduced cells with the addition of puromycin (Sigma) at a concentration of 1 µg/ml. Quantitative polymerase chain reaction (qPCR) was performed 72 hours after addition of puromycin to quantify the knockdown from each construct, and the stable cell lines that had the best knockdown was used for functional assays

Immunocytochemistry (ICC)

All steps were carried out at room temperature as described previously [38]. Briefly, cells were plated on poly-D-lysine-coated glass coverslips 48–72 hours post-treatment and were fixed in 4% (w/v) paraformaldehyde (Electron Microscopy Sciences) for 1 hour, washed in PBS (Invitrogen; 6 times, 5 minutes each), incubated for 1 hour in blocking buffer (PBS, 0.8% TritonX-100, 10% normal goat serum), and then incubated overnight in blocking buffer with anti-GABRA5 (1:100) (Aviva, ARP30687_P050) and anti-Bax (1:100) (Santa Cruz Biotechnology BAX C-7, SC-493) and anti-Bad (1:100) (Santa Cruz Biotechnology BAD C-7, SC-8044) antibodies. The cells were washed in PBS (6 times, 5 minutes each) before the addition of fluorescent goat anti-rabbit and goat anti-mouse secondary antibodies (Alexa Ig488 [green] or Alexa Ig565 [red], Invitrogen, Molecular Probes) at 1:250 for 60 minutes. The cells were washed in PBS (6 times, 5 minutes each) and visualized with a LSM700 confocal microscope (Zeiss, Oberkochen, Germany).

Immunocytochemistry (IHC)

Medulloblastoma tumors were cut to 14 microns thickness and air-dried over night. The slides were washed twice for 5 mins in TBS. 1%NaBH₄ in PBS was added for 30 mins, and then the slides were washed with 3XPBS. The slides were placed in a humidified slide box, and block was added to the slides, such that the slides were covered, and this was left for two hours. The block used was TBS+0.1%triton+5%Normalized goat serum. However, the block for primary and secondary antibody had 1% normalized goat serum. Rabbit polyclonal Aviva GABRA5 antibody was added to 1:100 dilution and 0.4 ml of the primary antibody containing block was added to each slide. The slides were covered with parafilm in a slide box at 4°C overnight. The next day, the slides were washed X3 for 20 mins in TBS. Secondary antibody 1:1000 dilution to the block for 2 h in the humidified chamber. The slides were then washed three times for 20 mins in TBS. The slides were dried, VectorMount, was added to the specimens prior to processing the slides for microscopy.

Western Blot

Membranes were blocked in PBS-Tween 5% milk and probed with either rabbit polyclonal anti-GABRA5 antibody (Aviva) or mouse monoclonal anti-p53 antibody (Santa Cruz Biotechnology DO1, SC-126) at the manufacturer's recommended concentrations. Anti-β-actin (Abcam, ab8227) was used as the protein-loading control. The horseradish peroxidase-conjugated secondary antibodies were used at 1:10 000 (Jackson ImmunoResearch Laboratories). Blots were developed using the SuperSignal West PICO Chemiluminescent Detection System (Pierce Biotechnology). Digital images were recorded using the Fuji Image 3000 Chemiluminescence Detection System (Fuji Film).

Cell Cycle Analysis

Cells were harvested 48 h after the addition of QH-ii-066, washed in PBS, and fixed with 70% ethanol overnight at 4°C. Cells were treated with 100 µg/mL of RNase A (Roche Diagnostics) and 50 µg/mL of propidium iodide (Sigma) for 30 minutes at 37°C in the dark. Propidium iodide fluorescence was monitored with FACSCalibur flow cytometry (BD Biosciences). At least 10 000 cells were collected and analyzed with Cell Quest Pro Software (BD Biosciences) and FlowJo software version 4.6.1 (TreeStar).

Electrophysiology

Whole-cell patch clamp recordings were made from medulloblastoma cell lines D556 and D458 at room temperature. These suspended cells were initially fixed on to coverslips coated with poly-D-lysine. Cell resting membrane potentials were measured with a standard potassium gluconate internal solution. GABA and QHii066 evoked currents were measured with cesium-based internal solutions. GABA (100 µM, dissolved in recording ACSF) and QHii066 (10 µM, dissolved in DMSO) were briefly (100–200 ms) puffed on the recorded neurons via a patch-type electrode using valve-controlled pressure application system (Picospritzer II). We allowed at least 25 s between GABA/QHii066 applications. Data were collected using an Axopatch 200B amplifier and Clampex 9.2 software (Axon Instruments) and filtered at 2 kHz, and digitized at 20 kHz using a Digidata 1320A.

Mouse xenografts studies

D425 medulloblastoma cells transfected with a firefly luciferase were prepared for implantation by collecting suspended cells and trypsinizing the remaining adherent cells. For intracranial xenografts, 4-week old mice were anesthetized with 0.2CC ketamine/xylacet (90mg/kg ketamine and 20mg/kg xylacet) and 200,000 luciferase-labeled medulloblastoma cells in a total volume of 2 µL were injected into the striatum over 5 minutes. The mice were allowed to recover from anesthesia and then returned to the animal facility, and were subsequently monitored by IVIS imaging at 1 week intervals using D-luciferin sodium salt administered at 150mg/kg. Seven days after intracranial injection, mice were separated into treatment cohorts which included DMSO control, QHii066 (50 mg/kg/d IP, days 14–21), or QHii066 + cisplatin (administered days 14–21, 50 mg/kg/d IP, and as single dose, 2 mg/kg, on day 13, respectively).

Results

GABR5 and α5-GABAA receptors are highly expressed and electrophysiologically active in Group 3 medulloblastomas

We analyzed gene expression microarray data from three previously published non-overlapping medulloblastoma cohorts and confirmed high levels of *GABR5* expression in Group 3 medulloblastomas (Figure 1a, $p < 0.0001$ in each dataset) [8,19,25]. We further verified expression of α subunit-containing GABAA receptors by immunofluorescence (IF) staining of formalin-fixed paraffin-embedded (FFPE) tumor samples, noting robust expression in a Group 3 medulloblastoma but no expression in a SHH-subtype tumor (Figure 1b). In addition, α5-GABAA receptor expression and staining pattern was maintained in patient-derived xenografts (flank and intracranial; see Figure 1b).

To identify whether the α5-GABAA receptor complexes were electrophysiologically active, we performed whole-cell patch clamp recording from GABR5 (+) medulloblastoma cells. Exposure to GABA (100µM) only evoked a small current (Figure 1c₁), however, much larger evoked currents were obtained in response to QHii066 (Figure 1c₂), a potent and specific agonist of α subunit-containing GABA-A receptors [21]. Whole-cell patch clamp

recording from GABR5 (–) DAOY medulloblastoma cells showed no evoked currents in response to QHii066 (Figure 1d₁). However, response to QHii066 was elicited when cells were co-transfected with GABR55/GABRB3/GABRG2 expression constructs (Figure 1d₂), highlighting the specificity of QHii066 for α subunit-containing receptor complexes. *Ex vivo* recordings in slice preparations from an intracranial xenograft established from a Group3/*MYC*-amplified GABR5 (+) medulloblastoma showed robust QHii066-evoked responses (Figure 1e, left panel) while no detectable QHii066-evoked responses were identified in neurons around tumor cells (Figure 1e, right panel; neuron identified by spontaneous synaptic responses). Notably, low reversal potentials were documented (~ -15 mV to -20 mV) from both *in vitro* and *ex vivo* recording, consistent with previous studies identifying significantly altered resting membrane potentials in cancer cells relative to cells from normal tissue counterparts [5].

Activation of $\alpha 5$ -GABAA receptors result in decreased viability and induction of apoptosis in medulloblastoma cells

We tested the effect of various agents targeting the GABAA receptor on cell viability in GABRA5(+), Group 3 medulloblastoma cells and observed a significant decrease in viability in response to QHii066 treatment (Figure 2a,b). Further supporting the on-target ($\alpha 5$ -GABAA receptor) effects of QHii066 but stable shRNA-mediated knockdown of GABRA5 mitigated the effect of QHii066 on medulloblastoma cell viability (Figure S1). In addition, QHii066 had little or limited effect on subventricular zone-derived normal human neural stem cells relative to the patient-derived MB002 cells (Figure S2). Analysis of cell viability over time and colony formation assays, both confirmed a cytotoxic effect of QHii066 on medulloblastoma cells (Figures 2c,S3). Immunofluorescence (IF) staining with anti-BAD antibody showed induction of this early apoptotic marker in response to QHii066 (Figure 2d), while flow analysis of propidium iodide stained cells showed an increase in sub-G0/G1 and accumulation of cells in S and G2 after treatment with QHii066 (Figure 2e). These results confirm induction of apoptosis and are consistent with responses noted in GABA-treated ES and neural crest stem cells where, in the absence of a G1 checkpoint and through induction of γ -H2AX, stem cells accumulate at the post-replicative S and G2 checkpoint [11,14].

Notably, GABA treatment alone or in combination with the positive allosteric modulator, diazepam, resulted in a mild decrease in cell viability. Muscimol, a potent GABA-A receptor agonist that binds the GABA binding site, which is distinct from the benzodiazepine (and QHii066) binding site, had no effect on medulloblastoma cell viability (data not shown). Similarly, phenobarbitone, a non-specific GABA agonist whose binding site is also distinct from the benzodiazepine binding site on the GABAA receptor complex, had an insignificant effect on cell viability (data not shown). No effect on cell viability was observed with GABAA receptor antagonists, picrotoxin and Xli093 [16], nor with the α -specific moderate inverse agonist, PWZ029 [27] (data not shown). Notably, concurrent treatment of medulloblastoma cells with a recombinant endozepine, diazepam binding inhibitor (DBI), blocked the effect of QHii066 on medulloblastoma cell viability (Figure S4), which is consistent with DBI's ability to block the inhibitory effect of GABAA receptor activation on neural progenitor cell proliferation in the subventricular zone [1].

QHii066 inhibits MYC- and TERT-associated transcriptional activity and induces HOX5-related gene expression

To identify downstream transcriptional events mediated by QHii066, we generated gene expression profiles of three medulloblastoma cell lines with increased *GABRA5* expression (D283, D425, MB002) and a medulloblastoma cell line (DAOY), which has neither expression of *GABR5* nor electrophysiological or biological response to QHii066 (Figure

3a). Of note, D425 and MB002 cells have high-level amplification of *MYC* [4, Bandopadhyay, manuscript in preparation] while the D283 medulloblastoma cells has been previously reported as having features consistent with both Group 3 [12] and Group 4 [29]. Gene set enrichment analysis (GSEA) [31] identified decreased *MYC*-related gene expression signatures in QHii066-treated GABRA5(+) medulloblastoma cells but not in control DAOY cells (Figure 3b). TERT-related transcriptional signatures were also inhibited consistent with previous studies establishing TERT as a direct transcriptional target of *MYC* (Figure 3c and Table S1) [39]. The top-scoring gene set enriched in QHii066-treated cells was a *HOXA5* transcriptional signature [7] (see Figure 3c,d and Table S2). *HOXA5* has been shown to suppress oncogenesis in several cancer types and its silencing/downregulation, usually by promoter hypermethylation, has been associated with poor prognosis in several malignancies [30]. To investigate the contribution of *HOXA5* to QHii066-mediated growth inhibition in medulloblastoma, we performed shRNA-mediated knockdown of *HOXA5* expression prior to QHii066 treatment. *HOXA5* knockdown substantially mitigated the response of medulloblastoma cells to QHii066 relative to scrambled control (Figure 3e).

QHii066 induces p53-dependent sensitization of medulloblastomas to cisplatin and radiation

HOXA5 has been shown to induce p53 expression through direct binding of four *HOXA5* binding elements in the p53 promoter [23]. Supporting this role, we observed increased p53 staining in medulloblastoma cells and by Western blot after treatment with QHii066 (Figure 4a,b). In addition, treatment with QHii066 substantially potentiated the effects of cisplatin while radiation exposure substantially sensitized cells to QHii066, both of which are used in current medulloblastoma treatment protocols (Figure 4c,d). Knockdown of p53 expression with shRNAs partially rescued cells from QHii066-mediated growth inhibition upon exposure to chemotherapy and radiotherapy (see Figure 4c,d). The *in vivo* efficacy of QHii066 as an antitumor agent was assessed in nude mice harboring orthotopic xenografts of the D425 *MYC*-amplified medulloblastoma cell line. A seven-day course of QHii066 (50mg/kg/d, intraperitoneal) resulted in a modest, though non-significant, increase in survival times relative to DMSO control treated mice. QHii066 in combination with the alkylating agent cisplatin (5mg/kg given on the day prior to QHii066) also resulted in a modest increase in survival relative to untreated mice (Figure 4e).

Discussion

Targeting of $\alpha 5$ -GABAA receptors in *MYC*-driven medulloblastomas with QHii066, a specific $\alpha 5$ -GABAA receptor agonist, results in decreased cell viability and sensitization to cisplatin and gamma irradiation. These effects are mediated in part by induction of *HOXA5* and subsequent upregulation of p53. Interestingly, the GABAA receptor $\alpha 1$ subunit has been shown to be repressed by *MYC* and is pro-apoptotic, and in this current study, QHii066 activates GABRA5, perhaps thereby causing global effects on *MYC* and p53 [35]. Whether this paradigm can be extrapolated to other cancers of neural and non-neural origin remains to be determined, but evidence implicates GABA pathway involvement in several other tumor types [2]. In neuroblastoma, *MYCN*-amplification has long been correlated with poor prognosis [18] and dysregulation of the GABA pathway has been reported as an independent predictor of clinical outcome [24]. Furthermore, *MYCN* and *GABRA5* expression were found to be significantly upregulated in neuroblastomas with high telomerase activity and poor prognosis [13]. Intriguingly, in lung squamous cell carcinomas, of the non-recurrent somatic mutations identified through whole exome/genome sequencing, the most frequently mutated group of genes are those involved with neuroactive ligand-receptor interactions with the fifth most common being “flumazenil pathway”, a gene set that consists primarily of GABA receptors and GABA pathway signaling components [6].

Further investigation into whether components of the GABA signaling axis can be leveraged for therapeutic purposes in these non-neural types of tumors will clarify the impact of this pathway more broadly across cancers.

Our study also presents several questions on “why” and “how” *GABRA5* is upregulated in medulloblastoma. The *GABRA5* genomic locus is highly regulated by DNA methylation and its dysregulation results in Prader-Willi/Angelman syndrome and other autism spectrum disorders [15]. Upregulated *GABRA5* expression in Group 3 medulloblastomas might reflect a globally dysregulated epigenome, and thus we could simply be leveraging “bystander” *GABRA5* expression for therapeutic gain. This is supported, in part, by the observation that stable shRNA-mediated knockdown of *GABRA5* did not overtly alter medulloblastoma cell growth, in vitro or in vivo. Nonetheless, the exact role of *GABRA5* in initiation or maintenance of medulloblastomas will need to be further clarified.

Similarly, GABA’s interface with the epigenome in the setting of cancer will be an area of significant interest. In embryonic stem cells and certain neural stem cell compartments, GABA signaling results in damage-independent phosphorylation/activation of H2AX through PI3K signaling and decreased proliferation. This effect is manifested by a prolonged transition through S/G2 and persistence of γ -H2AX foci, presumably in order to provide adequate genomic surveillance and repair of lesions introduced during mitosis [3,11]. Perhaps the marked chemo- and radiosensitization observed in medulloblastoma cells in response to QHii066 takes advantage of this phenomenon as the mutation burden introduced by cisplatin and radiation pushes cells towards apoptosis in lieu of repair. HDAC inhibitors have also been shown to prolong/enhance γ -H2AX loci formation after radiation, on the order of what was observed with QHii066-mediated chemo- and radiosensitization [41]. Supporting this correlation, our group and others have identified sensitivity of Group 3 medulloblastomas to multiple HDAC inhibitors, in vitro and in vivo (personal communication with Rob Wechlser-Reya). Whether these two mechanisms (HDAC inhibition and GABA receptor activation) can synergize to further sensitize medulloblastomas to endogenous and exogenous genotoxic stressors will be an important topic for future studies. In addition, as *Gabra5* is expressed in the dentate gyrus of the adult mouse, further studies will need to be carried out to assess any cognitive impacts of QHii066, with and without radiotherapy/chemotherapy, in the developing and mature brain [36].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. GABR5 is highly expressed and electrophysiologically active in Group 3 medulloblastomas

a, Box plots of *GABRA5* gene expression in three independent, non-overlapping medulloblastoma cohorts reveals high expression of GABR5 in 'Group 3' medulloblastomas. (*p*-values for one way ANOVA analyses between subgroups is shown for each cohort). b, Immunofluorescence staining reveals α -subunit expression is specific for Group 3 tumor (top left panel) and is not identified in a SHH subtype medulloblastoma (top right panel). GABR5 expression is preserved in a flank and intracranial xenograft derived from the same patient (bottom panels). c, Whole-cell patch clamp recording from GABR5 (+) D556 medulloblastoma cells shows small GABA-evoked currents (c₁) and much larger evoked currents in response to QHii066 (c₂), a potent and specific agonist of α subunit-containing GABA-A receptors. Recordings were performed at various holding potentials as shown. Representative differential interference contrast (DIC) images are shown in the top panel. d, Whole-cell patch clamp recording from GABR5 (-) DAOY medulloblastoma cells shows no evoked currents in response to QHii066 (d₁), but response to QHii066 is elicited when cells are co-transfected with GABR55-GABRB3/GABRG2 (d₂), showing the specificity of QHii066 for α subunit-containing receptor complexes. Representative DIC images are shown in the top panel. e, QHii066-evoked responses in a slice preparation from an intracranial xenograft established from a MYC-amplified, GABR5 (+) medulloblastoma at different holding potentials (right panel). No detectable QHii066-evoked responses were identified in neurons around tumor cells (left panel; neuron identified by spontaneous synaptic responses).

Figure 2. Activation of α 5-GABAA receptor with QHii066 results in decreased viability and induction of apoptosis in medulloblastoma cells

a, Quantitative RT-PCR of GABR5 across established medulloblastoma cell lines show correlation of GABR5 expression with presence of MYC-amplification/Group 3 status. b, Dose-response curves of medulloblastoma cells to QHii066 using MTS cell viability assay show QHii066-mediated decrease in viability in a GABR5 dependent manner. c, Clonogenic colony formation in soft agar is inhibited by QHii066. (**p < 0.0001). d, Immunofluorescence staining with anti-BAD antibody shows induction of apoptosis in MYC-amplified medulloblastoma cell line D425 treated with QHii066. e, Propidium iodide staining and flow analysis show accumulation of D425 cells in sub-G₁ and G₂-M upon treatment with QHii066.

Figure 3. Chemical genomic profiling of QHii066 confirms inhibition of MYC-associated transcriptional activity and reveals enrichment of HOXA5 signatures

a, Heatmap representation of the top 50 down- and upregulated genes (top panel; $p < 0.0001$) and selected genes (bottom panel) following QHii066 treatment in three independent MYC-amplified, GABR5 (+) medulloblastoma cell lines. MYC (red arrow) and GABRA5 are both downregulated by QHii066 treatment. Data are presented row normalized (range -3 to 3 standard deviations from median in expression). b, Enrichment plots showing inhibition of MYC-related gene sets. c, Table of top scoring gene sets in QHii066-treated medulloblastoma cells and DMSO-treated control cells. d, Enrichment plot of HOXA5 gene set in QHii066-treated cells relative to DMSO control. e, shRNA-mediated knockdown of HOXA5 decreases sensitivity of medulloblastoma cells to QHii066.

Figure 4. QHii066 induces p53 expression and sensitizes medulloblastoma cells to cisplatin and radiation

a, Immunofluorescence staining of QHii066-treated Group 3 D425 medulloblastoma cells shows increased p53 after QHii066 treatment. b, Western blot confirms increase in p53 after treatment of D425 medulloblastoma cells with QHii066 (2.5 uM) and effective decrease in p53 protein levels after shRNA-mediated knockdown. c, MTS cell viability assay of medulloblastoma cells treated with a 1 Gy dose of radiation and increasing doses of QHii066 show increase in sensitivity to QHii066 which is ablated upon p53 knockdown. d, MTS survival assay of medulloblastoma cells treated with 1.25 uM dose of QHii066 and increasing doses of cisplatin show marked shift in IC₅₀ in presence of QHii066, again ablated by knockdown of p53. e, Kaplan-Meier survival analysis of QHii066 +/- cisplatin treated mice harboring orthotopic D425 medulloblastoma xenografts. 200,000 medulloblastoma cells were injected into the striatum of 4-week old mice and stratified into groups treated with DMSO control, QHii066 (50 mg/kg/d IP, days 14–21; Log-rank Mantel-Cox test p-value = 0.104), or QHii066 + cisplatin (administered days 14–21, 75 mg/kg/d IP, and as single dose, 5 mg/kg, on day 13, respectively; Log-rank Mantel-Cox test p-value = 0.0646).