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Metabolic Reprogramming by Dual AKT/ERK Inhibition Through Imipridones Elicits Unique Vulnerabilities in Glioblastoma

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Abstract

Purpose: The goal of this study is to enhance the efficacy of imipridones, a novel class of AKT/ERK inhibitors that displayed limited therapeutic efficacy against glioblastoma.

Experimental Design: Gene set enrichment, LC/MS and extracellular flux analysis were used to determine the mechanism of action of novel imipridone compounds, ONC206 and ONC212. Orthotopic patient-derived xenografts were utilized to evaluate therapeutic potency.

Results: Imipridones reduce the proliferation of patient-derived xenograft and stem-like glioblastoma cell cultures *in vitro* and in multiple xenograft models *in vivo*. ONC212 displayed the highest potency. High levels of c-myc predict susceptibility to growth inhibition and apoptosis induction by imipridones and increased host survival in orthotopic patient-derived xenografts. As early as 1h, imipridones elicit on-target inhibition, followed by dephosphorylation of GSK3 β at serine 9. GSK3 β promotes phosphorylation of c-myc at threonine 58 and enhances its proteasomal degradation. Moreover, inhibition of c-myc by BRD4 antagonists sensitizes for imipridone induced apoptosis in stem-like GBM cells *in vitro* and *in vivo*. Imipridones affect energy metabolism by suppressing both glycolysis and oxidative phosphorylation, which is accompanied by a compensatory activation of the serine-one carbon-glycine (SOG) pathway, involving the transcription factor ATF4. Interference with the SOG pathway through novel inhibitors of PHGDH results in synergistic cell death induction *in vitro* and *in vivo*.

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Conclusion: These results suggest that c-myc expression predicts therapeutic responses to imipridones and that imipridones lead to suppression of tumor cell energy metabolism, eliciting unique metabolic vulnerabilities that can be exploited for clinical relevant drug combination therapies.

Introduction

Imipridone derivatives are a new class of molecules that have entered clinical testing for solid and non-solid malignancies. Representative examples are the lead compound, called ONC201 (1) and the chemically modified and presumably enhanced derivatives, ONC206 and ONC212 (2). The original compound, ONC201, was identified in a screen for molecules that induce TRAIL, a cytokine that in the field of oncology was considered as the holy grail for cancer therapy since it specifically kills tumor cells in a rapid manner, but not non-neoplastic cells (1). On the molecular level, ONC201 elicited an increase of TRAIL transcription through a FOXO3A dependent mechanism, involving the inhibition of the kinases ERK and AKT (1). Early after the discovery of ONC201, it became evident that this compound exerts additional anti-cancer properties, e.g. the inhibition of proliferation in cells that do not undergo apoptosis upon ONC201 administration (3–5). These additional features are in part attributed to the fact that imipridones inhibit dopamine receptor signaling, such as DRD2 (6).

Tumor cell metabolism has become a central focus in cancer research since it bears the potential to target metabolic aberrancies in tumors, thus providing a therapeutic window. Although cancer cells often depend on glycolysis in the presence of abundant oxygen (called Warburg effect), it has become evident that other metabolic pathways are targetable in malignant cells, even oxidative phosphorylation (OXPHOS), the most efficient form of energy generation in cells. Quite prominent is the metabolic dependency on glutamine by many malignant tumors (7–9), which requires the presence of a functional OXPHOS. Being a master regulator of tumor stem cells, the transcription factor c-myc (10–12) is at the core of regulation of tumor cell metabolism and affects the expression of enzymes related to glycolysis, glutamine and OXPHOS.

In this report, we provide evidence that novel imipridones with different potency are highly effective against state of the art-model systems of glioblastoma, involving in particular glioma stem-like cells. We have unraveled a novel mechanism by which imipridones elicit their anti-glioma activity, involving suppression of the master regulator, c-myc, as early as 1h and shut down of tumor energy cell metabolism, leading to a state of energy deprivation and tumor cell cytoskeleton and apoptosis. Moreover, high-levels of c-myc predict both apoptotic response and inhibition of proliferation of GBM cells to imipridone derivatives and extended host survival in orthotopic patient-derived xenografts of glioblastoma, positioning c-myc as a potential therapeutic predictive marker, and that further inhibition of c-myc along with imipridones induces synergistic growth reduction *in vitro* and *in vivo*. In addition, we have unraveled that upon imipridone induced energy deprivation the serine-one carbon-glycine pathway is increased, eliciting a novel unique metabolic vulnerability.

Materials and Methods

Reagents

Imipridones, ONC201, ONC206 and ONC212, were received from Oncoceutics. OTX015 was purchased from Selleckchem (Houston, TX). A 10 mM working solution in dimethylsulfoxide (DMSO) was prepared for all reagents prior to storage at -20°C . Final concentrations of DMSO were below 0.1% (v/v). The plasmids for wild-type c-myc and mutant c-myc were obtained from Addgene (ID: 45597 and 45598).

Cell cultures and growth conditions

All cells were cultured as described (13–17). The identities of the cell cultures were confirmed by the respective source of purchase. LN229 and U87 cells are established glioblastoma cells. SF188 is a *MYC* amplified pediatric glioblastoma cell culture. NCH644, NCH690 and NCH421K glioma stem-like cells were cultured in MG-43 medium (CLS, Heidelberg, Germany) for both maintenance and experiments (13–15,18). GBM12 and GBM14 are patient derived xenograft tumors as described elsewhere (13–17). Human astrocytes were obtained from ScienCell Research Laboratories, Inc. and cultured as recommended by the provider.

Cell viability assays

In order to examine cellular proliferation, CellTiter-Glo® assays were performed as previously described. ATP levels were determined as performed in (13–17).

Measurement of apoptosis and mitochondrial membrane potential

Annexin V/propidium iodide, propidium iodide and TMRE staining (for mitochondrial membrane potential) were performed as previously described (13–17) or in accordance with the manufacturer instructions for TMRE staining (Cell Signaling). The data were analyzed with the FlowJo software (version 8.7.1; Tree Star, Ashland, OR).

Extracellular flux analysis

Extracellular flux analysis was performed on the Seahorse XFe24 Analyzer. The “XF cell mito stress test kit” (Agilent Technologies) was utilized to determine parameters relevant to oxidative phosphorylation and determined as described earlier in (19). GBM cells were incubated with Seahorse XF base medium supplemented with 5 mM glucose, 1 mM pyruvate, and 2 mM L-glutamine in a CO₂-free incubator for 1h before the assay. During the course of the assay, 2 μM oligomycin, 2 μM FCCP, and 0.5 μM rotenone/antimycin were added sequentially. Regarding glycolysis, the “XF cell glycolysis stress test kit” (Agilent Technologies) was used in accordance with manufacturer’s instructions. GBM cells were incubated with Seahorse XF base medium supplemented with 1 mM L-glutamine in a CO₂-free incubator for 1h before the assay. During the course of the assay, 10 mM glucose, 1 μM oligomycin, and 50 mM 2-DG) were added sequentially.

Liquid chromatography/mass spectrometry (LC/MS) analysis of metabolites

Metabolite analysis was carried out on a [Thermo Scientific QExactive Orbitrap](#) in a manner as described earlier by others (20).

Western blot analysis and capillary electrophoresis on Wes instrument (Proteinsimple)

Specific protein expression in cell lines was determined by Western blot analysis or capillary electrophoresis as described before. Capillary electrophoresis was run on the Wes instrument (Proteinsimple, CA). The following antibodies were used on the Wes instrument: p-Akt (serine 473) (1:25, CST, Cell Signaling Technology, Danvers, MA), Akt (1:50, CST), Mcl-1 (1:50; CST), Bcl-2 (1:25; R&D Systems), BIM (1:25; CST), Bcl-xL (1:25; CST), c-myc (1:25, CST), Usp9X (1:25; CST), Noxa (1:25, clone 114C307; Calbiochem), p-Akt (1:25, CST), Akt (1:25, CST), p-AMPK (1:25, CST), AMPK (1:25, CST), PHGDH antibody (Novus, #NBP1-87311), PSAT1 Polyclonal Antibody (Invitrogen #PA5-22124), β -actin (1:250, clone AC15; Sigma Aldrich) and secondary HRP-linked antibodies were purchased from Santa Cruz Biotechnology Inc. For the expression levels of respiratory complexes, the Total OXPHOS Human WB Antibody Cocktail was used (Abcam, Cambridge, MA). Western blots were acquired, using the Azure (C300) imaging system (CCD – camera based).

Real-time PCR analysis

RNA was isolated and reverse-transcribed as previously described (21). For cDNA amplification, c-myc primers were used: Forward: CCT GGT GCT CCA TGA GGA GAC Reverse: CAG ACT CTG ACC TTT TGC GAG G. Amplification of 18S served as normalization control. For the determination of mtDNA, the following primers were used: Forward cga aag gac aag aga aat aag g; Reverse: ctg taa agt ttt aag ttt tat gcg. The other primers were designed by Origene Technologies.

Microarray and gene set enrichment analysis (GSEA)

Transcriptome analysis and GSEA, involving microarrays, was performed as previously described in (21). The related data and cel files are archived through GEO under the following accession numbers: GSE104273 and GSE103963.

Transfections of siRNAs or transductions of shRNAs

Transfections were performed as previously described (22), using either Oligofectamine or Lipofectamine 2000. CMYC siRNA 1 and 2 were purchased from Cell signaling. DRD2 specific siRNAs were obtained from DharmaconTM. Non-targeting siRNA-pool (ON-TARGETplus Non-targeting Pool, # D-001810-10-20), was purchased from DharmaconTM. Lentiviral shRNA particles targeting c-myc were purchased from Santa Cruz Biotech.

Orthotopic xenograft models of glioblastoma

The individual cells were injected as previously described in (16,21). Briefly, fifty thousand U87 cells or 3×10^5 GBM123 cells in 2 μ L of PBS were intracranially injected into nu/nu mouse. After 3 days of engraftment, the drug treatments were initiated. All treatments were administered by intraperitoneal injection (i.p.).

Subcutaneous xenograft model

NCH644 (GBM stem-like cells), U87-EGFRvIII or HCT116 (colon carcinoma) cells were suspended 1:1 in Matrigel® Matrix (Corning Inc., Corning, NY) and implanted subcutaneously into the flanks of 6–8 week-old Nu/Nu mice. Tumors were measured with a caliper and sizes calculated according to the standard formula: $(\text{length} * \text{width}^2) * 0.5$. All treatments were administered by intraperitoneal injection (i.p.). Drug compounds were dissolved in 10% DMSO, 32% Cremophor EL (SIGMA, St. Louis, MO), 8% Ethanol (Pharmco-Aaper, Brookfield, CT) and 50% PBS.

Immunohistochemistry

PDX xenografted tumors were fixed in formalin and embedded in paraffin as previously described (13). Paraffin sections were cut and stained with Hematoxylin and Eosin or subjected to immunohistochemistry for TUNEL or Ki67 staining as described (13).

Statistical analysis

Statistical significance was assessed by two-tailed Student's t-test or the Mann-Whitney test using Prism version 5.04 (GraphPad, La Jolla, CA). A $p < 0.05$ was considered statistically significant.

Study approval

All procedures were in accordance with Animal Welfare Regulations and approved by the Institutional Animal Care and Use Committee at the Columbia University Medical Center.

Results

High levels of c-myc protein predict apoptosis induction and inhibition of proliferation by imipridones in model systems of GBM

The chemical structures of the different imipridones are shown in Figure 1A. Therein, ONC201 represents the lead compound, whereas ONC206 and ONC212 are chemically modified derivatives with anticipated enhanced efficacy (Figure 1A). To get an understanding of the underlying cause of the observed reduction in cellular viability, we conducted cell cycle analysis of GBM cultures after treatment with various imipridones for 72h. Our observations suggested that GBM cells with high c-myc levels were more likely to respond with apoptosis induction upon imipridone (ONC201, ONC206 and ONC212) treatment (Figure 1B-G and Supplementary Figure 1F). While in patient-derived xenograft GBM14 cells, established LN229, T98G and U87 GBM (lower levels) cells we only observed a settle increase in sub-G1 (apoptotic) cells (Figure 1B and 1C), this effect was more pronounced in stem-like NCH644, NCH690 and NCH421k cells as well as in a pediatric GBM cell line, SF188 (all very high c-myc expression), (Supplementary Figure 1 and Figure 1B and 1E-G). Consistent with the highest expression levels of c-myc, NCH421k glioma stem-like cells underwent apoptosis most strikingly upon administration of imipridones (Figure 1B, 1E, 1G and Supplementary Figure 1C). Similarly, NCH690 glioma stem-like cells treated with various imipridones displayed a high fraction of Annexin V positive cells (Supplementary Figure 1D and 1B), which occurred in a time-dependent

manner (Supplementary Figure 1A-C). Notably, c-myc suppression precedes apoptosis induction in NHC421k cells (Supplementary Figure 1A-C). Among imipridones, ONC212 (nano molar range) was most potent to elucidate these changes when compared to ONC201 (micro molar range) (Figure 1B, 1G Supplementary Figure 1). However, despite the fact that intermediate c-myc expressing cells (U87) or low c-myc expressing cells (GBM14), did not undergo apoptosis, they still display inhibition of proliferation (IC_{80} values) upon imipridone administration in accordance with their expression level of c-myc (Figure 1E and 1G). Notably, non-neoplastic human astrocytes displayed the highest resistance against imipridones (Figure 1G).

We modulated the expression levels of c-myc in an intermediate expressing c-myc line, U87, as well as in *MYC*-amplified SF188 cells (very high levels). We confirmed that SF188 cells are acutely dependent on c-myc for their survival. To test this hypothesis, we acutely silenced the expression of c-myc by two c-myc targeting siRNAs and subsequently analyzed them for apoptosis induction (Supplementary Figure 1E). Our findings indicate that silencing of c-myc elicited significant apoptosis induction in these cells, reinforcing their dependence on the transcription factor, c-myc. Next, we created a stable cell c-myc overexpressing U87 line and evaluated its susceptibility to ONC201 in the context of apoptosis induction (Figure 1H and 1I). In agreement with our hypothesis, we found that c-myc overexpressing cells displayed a higher rate of DNA – fragmentation upon imipridone treatment as compared to control transduced cells. In a reciprocal approach, we created a stable SF188 line with silenced c-myc expression (lentiviral shRNA) that in turn becomes less addicted to c-myc. As anticipated, SF188 GBM cells with silenced c-myc expression were less sensitive to apoptosis induction by imipridones as compared to cells transduced with a non-targeting shRNA (Figure 1J and 1K). These findings suggest that imipridones mediate their effects in part by inhibition of proliferation and apoptosis induction in a cell-type and c-myc dependent manner.

Imipridones regulate phosphorylation of AKT, ERK and FOXO3A, mTORC1 signaling, expression of anti- and pro-apoptotic Bcl-2 family members and c-myc protein levels in GBM model systems

To assess the pharmacodynamics properties of imipridones in model systems of GBM, we performed protein capillary electrophoresis for the expression of phosphorylated Akt, total Akt, phosphorylated ERK, total ERK, phosphorylated FOXO3A (down-stream targets of AKT and ERK) and total FOXO3A in U87 and stem-like GBM cells, NCH644 after treatment with ONC201, ONC206 and ONC212 at 200nM for 72h (Figure 2A and Supplementary Figure 2A). While, as anticipated, ONC201 did not show activity, ONC212 was most potent to affect the phosphorylation status of FOXO3A, Akt and ERK, in keeping with the cellular viability assays. However, higher concentrations of ONC201 resembled the above findings related to ONC212. Next, we assessed the activation status of mTORC1 signaling after imipridones exposure to U87 and stem-like GBM cells, NCH644 because earlier findings suggested that ONC201/TIC10 interfered with this signaling cascade. We found that mTORC1 targets, mTOR, 4EBP1, S6K, S6, were most potently and consistently inhibited by ONC212 in U87 and NCH644 stem-like GBM cells (Figure 2A and Supplementary Figure 2A).

To determine if the potency of imipridones correlates with the regulation of apoptosis mediators (Bcl-2 family proteins) (13), including their chaperones, Usp9X and Bag3, we assessed as to whether or not ONC201 derivatives, ONC206 and ONC212, would regulate the protein levels of those molecules in a more potent manner. We found that in U87 and stem-like GBM cells, NCH644, ONC206 and ONC212 at 200 nM suppressed the expression levels of anti-apoptotic, Bcl-2, Bcl-xL and Mcl-1, and the deubiquitinase Usp9X, while in contrast at the same concentration ONC201 was less effective (Figure 2B and Supplementary Figure 2B). Under these conditions, pro-apoptotic Bcl-2 family members, BIM and Noxa were down regulated after ONC206 and ONC212 treatment (Figure 2B and Supplementary Figure 2B). However, ONC201 (10 μ M) up-regulated BIM in U87 cells, priming cells for potential pro-apoptotic stimuli (Figure 2B and Supplementary Figure 2B). In essence, these findings support the idea that imipridones convert the cells into a more “apoptosis favorable” state.

Imipridones downregulate protein levels of the transcription factor, c-myc, at the posttranslational level

We found substantial evidence for suppression of c-myc downstream targets by imipridones in accordance with their potency (Figure 2C). We conducted a time course experiment to assess c-myc protein levels and detected a down-regulation of c-myc as early as 2h and extending to 72h after imipridone treatment in established and stem-like GBM cells (Figure 2D and 2H). Of note, ONC212 had the strongest impact on c-myc levels, consistent with its strongest potency amongst imipridones. We hypothesized that based on our transcriptome analysis most likely imipridones modulate the stability of c-myc protein since this transcription factor is known to have a very short half-life and is highly susceptible to proteasomal degradation upon phosphorylation at threonine 58. Therefore, we conducted a time course analysis of phosphorylated and total c-myc protein levels upon stimulation with imipridones (Figure 2H). While ONC201 led to a rapid depletion of total c-myc protein (within 2h) and decrease of phosphorylated c-myc at serine 62, it increased phosphorylation of c-myc at threonine 58, which was preceded and coincided with a reduction in phosphorylation of GSK3 β at serine 9, a kinase that is known to phosphorylate c-myc at threonine 58 (Figure 2H) (23,24). Given that GSK3 β is a substrate of AKT, we assessed AKT phosphorylation status and found that ONC201 decreased phosphorylation of AKT at serine 473, coinciding with GSK3 β dephosphorylation (Figure 2H). Consistently, two pharmacological inhibitors of GSK3 β restored c-myc protein levels upon imipridone treatment (Figure 2I), confirming the pivotal role of GSK3 β in imipridone mediated reduction of c-myc protein levels. Moreover, imipridones did not suppress protein levels of a T58A c-myc mutant (a hotspot region for mutations), further supporting the role of GSK3 β in the mechanism (Figure 2J). To confirm that these phosphorylation events of c-myc result in a decrease of c-myc stability, U87 GBM cells were treated with ONC212 or DMSO and exposed to protein synthesis inhibitor, cycloheximide. ONC212 reduced the stability of c-myc protein (Figure 2E and 2F). Next, we treated U87 GBM cells with ONC212 in the presence or absence of the bona-fide proteasomal inhibitor, MG132. In keeping with the initial hypothesis, MG132 attenuated ONC212 mediated suppression of c-myc protein (Figure 2G). Similar findings were made with the lead compound ONC201 (Supplementary Figure 2D, 2E). To determine the relative contribution of c-myc mRNA suppression by

imipridones, we treated U87GBM cells with various concentration of ONC201, ONC206 and ONC212 and assessed c-myc mRNA levels. We found only slight suppression of c-myc mRNA levels, suggesting that the major effects of imipridones on c-myc is non-transcriptional (Supplementary Figure 3A and 3B). Overall, these findings suggest that imipridone enhance c-myc degradation, most likely through regulation of the proteasomal pathway, involving GSK3 β mediated phosphorylation of threonine 58.

Next, we posed the question as to whether or not simultaneous targeting of c-myc along with imipridones provides a potential therapeutic benefit. To this end, we utilized a bromodomain protein inhibitor (BRD2–4), OTX015, a compound that has been shown to transcriptionally antagonize the expression of c-myc (25,26) and tested the efficacy of imipridones in the presence or absence of this inhibitor in stem-like GBM cells, NCH644 and NCH421k. NCH644 and NCH421k cells were treated with OTX015, imipridones or the combination and analyzed for apoptosis by Annexin V/PI staining with subsequent flow cytometry. In the presence of OTX015, imipridones induced apoptosis was significantly enhanced (Supplementary Figure 2C), suggesting that dual-targeting c-myc by BRD4 inhibitors and imipridones might be viable treatment strategy that is effective against tumor initiating cells.

Imipridones reprogram the transcriptome of GBM cells and suppress glycolysis and oxidative phosphorylation

In order to identify the underlying mechanisms by which imipridones elicit their anti-glioma effects, we conducted a transcriptome analysis and subsequent gene set enrichment analysis of GBM cells treated with ONC201, ONC206 and ONC212 (at 200 nM) as well as ONC201 at 10 μ M. In keeping with the western blot findings, we only identified transcriptomic changes in cells treated with ONC206 and ONC212 at 200 nM, while ONC201 appeared to elicit no effects at that concentration (not shown). However, higher concentrations of ONC201 (10 μ M) resembled the effects of ONC206 and ONC212. Our GSEA analysis uncovered additional mechanistic insights in the biology of imipridones. Most notably, we found changes related to cellular metabolism that suggest that imipridones suppress glycolysis as well as oxidative phosphorylation, resulting in a “transcriptional state of energy starvation” (Figure 3A). These findings were linked to changes in the expression of relevant associated transcription factors. As mentioned earlier, we identified several gene sets to be downregulated related to c-myc targets (Supplementary Figure 3A), suggesting that first c-myc is likely a key player in imipridone mediated anti-glioma activity and that second c-myc is likely responsible for the transcriptional metabolic reprogramming of GBM cells by imipridones since many of the c-myc targets are genes implicated in metabolism.

In light of these transcriptional findings, we hypothesized that imipridones affect ATP levels of tumor cells. Our results suggest that ONC201 and ONC206 reduce ATP levels in a dose dependent manner (Figure 3B). Notably, ONC212 was most efficacious to reduce ATP levels, followed by ONC206 and ONC201 (Figure 3B). Accompanied by the decline of ATP levels, we found an increase of phosphorylation of the AMP sensitive kinase, AMPK (threonine 172), starting as early as 24 hours (Supplementary Figure 3C). In keeping with the ATP levels, ONC212 was most potent in enhancing the phosphorylation of AMPK. It is generally accepted that low energy levels are causal for the accumulation of unfolded

proteins in the endoplasmic reticulum. In keeping with that notion, we found evidence of activation of several ER-stress related pathways as manifested by elevation of IRE1 α , ATF4, ATF3 and phosphorylated eif2 α proteins (Supplementary Figure 3D).

Since the global transcriptome analysis suggests an inhibition of glycolysis and oxidative energy metabolism (Supplementary Figure 4A-E), we conducted extracellular flux analysis. To this purpose, SF188 (high c-myc) and U87 (intermediate c-myc levels) GBM cells were treated with vehicle, ONC201, ONC206 or ONC212 and assayed for glycolytic activity by extracellular flux analysis (Figure 3C-E, Supplementary Figure 4E-4L and 5A-E). We found that imipridones, ONC206 and ONC212, were most potent to suppress baseline extracellular acidification rate (ECAR) (Supplementary Figure 5A-E), which was more pronounced in high c-myc expressing SF188 cells as compared to U87 (Figure 3E). However, utilizing 10 μ M of the lead compound, ONC201, recapitulated the effects of ONC206 and ONC212 on glycolysis (Supplementary Figure 4F-I). In addition, we tested whether or not c-myc overexpressing U87 cells are more susceptible to glycolysis inhibition by imipridones. As shown in Figure 3F, c-myc overexpressing cells are more susceptible to ECAR reduction as compared to vector transduced cells. We assessed the protein levels of glycolysis related enzymes and transporters, HK2, LDHA and GLUT1. To this purpose, U87 cells were treated with various imipridones as above. We found that ONC206 and ONC212 reduced the levels of HK2, GLUT1 and LDH (Figure 3G). In order to confirm that imipridone mediated suppression of c-myc is sufficient to affect glycolysis, we performed knockdown experiments, involving two c-myc siRNA oligonucleotides. There was a siRNA dependent reduction in c-myc protein levels and an associated depletion of HK2, LDHA and GLUT1, in keeping with the hypothesis that c-myc governs the expression of these proteins and that this is also true in the setting of GBM model systems (Figure 3). To further delineate the role of c-myc in glycolytic metabolism, we pharmacologically inhibited c-myc by using OTX015. We have previously shown that the concentrations of OTX015 applied in the current studies were sufficient to suppress c-myc protein levels (10,27). Using this dosage of OTX015, we performed extracellular flux analysis. We found that c-myc inhibition recapitulates the effects on ECAR mediated imipridones, further more suggesting the pivotal role of c-myc in imipridone mediated glycolytic inhibition (Figure 3I). To further assess the impact of imipridones on glycolysis, we measured glycolytic metabolites by LC/MS following ONC212 treatment. These results show that ONC212 leads to an accumulation of glycolytic metabolites, suggesting a glycolytic block with an overall reduction of glycolysis (as supported by the extracellular flux analysis with reduction in ECAR) (Figure 3J).

Next, we determined the effects of imipridones on OXPHOS related metabolism. To this end, U87 GBM cells were treated with vehicle, ONC201, ONC206 and ONC212 for 24 h and analyzed for basal oxygen consumption rate, ATP production and maximal respiration (Figure 4A-C and Supplementary Figure 5F). We detected a significant reduction in all these parameters in cells treated with 200 nM ONC206 and ONC212 (Figure 4A-C and Supplementary Figure 5F). In contrast, 200 nM of ONC201 had little effect on mitochondrial respiration (Figure 4A-B). However, 10 μ M of ONC201 recapitulated the effects of ONC206 and ONC212 (Figure 4A-C and Supplementary Figure 5H), further corroborating the earlier notion that ONC206 and ONC212 are more potent than ONC201. We assessed the relative potency of imipridone OCR reduction in the context of high and

intermediate c-myc expression. As anticipated, high c-myc expressing SF188 cells were more prone to OCR inhibition by imipridones as compared to U87 cells (Figure 4C).

Given the significant suppression of OXPHOS, we evaluated the underlying mechanisms governing these changes. We conducted western blot analysis to detect the expression levels for respiratory complexes, pyruvate dehydrogenase (PDH). To this purpose, U87, LN229 and stem-like GBM cells, NCH644, were treated with vehicle, ONC201, ONC206 and ONC212 for 72h (Figure 4D and Supplementary Figure 5H and 5I). While no effects on the expression of respiratory complexes were detected with 200 nM of ONC201, the imipridone derivatives, ONC206 and ONC212 suppressed the levels of OXPHOS complexes as well as of PDH at that concentration (Figure 4D). However, 10 μ M of ONC201 suppressed OXPHOS complexes as well (Figure 4D and Supplementary Figure 5H and 5I). Similarly, c-myc knockdown also affected the protein expression of respiratory complexes (Figure 4E-F), in keeping with the effects of imipridones. To assess the functional implication of c-myc on OXPHOS in glioblastoma cells, U87 GBM cells were treated with OTX015 for 24 h and subsequently analyzed by extracellular flux analysis. OTX-015 reduced the oxygen consumption rate (Figure 4G). Given the suppression of OXPHOS, we analyzed TCA metabolite levels upon treatment with imipridone derivatives by LC/MS. In keeping with the findings obtained with the extracellular flux analysis, we encountered a suppression of TCA cycle intermediates (Figure 4H), further corroborating the earlier notion that imipridones significantly impair tumor energy metabolism. Consistent with an impact on mitochondrial metabolism, we found a significant suppression of mtDNA levels in imipridone treated U87 GBM cells, which is most pronounced in ONC212 treated cells (Figure 4I). Similarly, mitochondrial membrane potential was lowered by ONC212 (Supplementary Figure 5G).

Imipridones enhance serine-one carbon-glycine metabolism

While imipridones suppress the main energetic metabolic pathways of GBM cells, we were hypothesizing that compensatory pathways will be activated. In this vein, GSEA provided information that imipridone treated GBM cells activate the serine-one carbon-glycine metabolism with an increase of *PHGDH*, *PSAT1* and *PSPH* and other enzyme related genes to the folate cycle (Figure 5A and Supplementary Figure 6A). These findings were confirmed by real-time PCR and protein expression analysis in both glioblastoma and colonic carcinoma cells, suggesting that these observations are not limited to just GBM (Figure 5B and 5D and Supplementary Figure 7A). Consistently, we found that depriving GBM cells of energy (ATP) by the ATP synthase inhibitor, oligomycin, up-regulates *PHGDH* and *PSAT1*, in keeping with our hypothesis that energy deprivation leads to the activation of this pathway (Supplementary Figure 6D). To corroborate these findings, LC/MS analysis was performed, which showed significant increases of metabolites related to the serine-one carbon-glycine metabolism in imipridone treated cells (Figure 5C). To address the question by which mechanism imipridones induce this pathway, we consulted our GSEA data and the literature and concluded that likely ATF4 is the pivotal mediator of these transcriptional changes. To test this hypothesis, we silenced ATF4 levels in the presence or absence of imipridones and found that ATF4 silencing (involving one siRNA pool, four distinct siRNAs and a shRNA construct) attenuates imipridone mediated increase of *PHGDH* and *PSAT1* (Figure 5E, Supplementary Figure 6B, 6C and Supplementary

Figure 7B and 7C). Next, we determined the translational impact of pharmacological targeting of the SOG pathway along with imipridones. To this end, we used two recently identified pharmacological inhibitors of PHGDH, NCT-503 and CBR-5884 (28), and combined them with ONC212. We found that the combination treatment of NCT-503 and ONC212 enhanced induction of apoptosis more potent than each compound alone in stem-cell like GBM cells, NCH421k, NCH644 and NCH690, as well as in U87 GBM cells (Figure 5F). Similar results were obtained when we administered CBR-5884 in lieu of NCT-503. These synergistic apoptotic effects were not limited to glioblastoma model systems since HCT116 colon carcinoma cells showed a comparable response rate to the combination treatment (Supplementary Figure 7D). The findings of the activation of the SOG – pathway are summarized in Supplementary Figure 7G.

The novel imipridone derivatives are active either singly or in combination therapies in multiple xenograft models and extend host survival in orthotopic patient-derived xenografts of GBMs

PDX model systems remain the most relevant preclinical model systems to predict anti-tumor responses in patients, which is accepted in the studies of GBM as well. Following this strategy, we compared the efficacy of the lead compound, ONC201, with the chemical derivative, ONC212, which was the most potent in all assays related to *in vitro* testing. To this purpose, we used two PDX models, GBM12 and GBM43 that have different mutational backgrounds and susceptibilities to the standard of care treatment (temozolomide) (29). These lines were implanted in immunocompromised mice and after tumors were established three treatment groups were formed, consisting of vehicle, ONC201 and ONC212. Treatment was performed two times a week. Unanimously, we found that ONC212 was most potent in reducing tumor growth (Supplementary Figure 8A-F), intimating that imipridones are efficacious in relevant preclinical animal models of GBM and that chemical modification results in an improvement of efficacy of imipridones. Finally, to account for all here studied imipridones, we conducted a third animal model, using the highly challenging to treat U87-EGFRvIII model. Tumor cells were implanted subcutaneously and upon establishment of the tumors, three groups were formed for subsequent treatments with vehicle, ONC206 and ONC212. Upon conclusion of the study, host tumor sizes in animals treated with ONC212 were the smallest as compared to vehicle or ONC206 (Supplementary Figure 8G-I), in keeping with the results in the PDX models. To account for toxicity, we measured the weights of the animals and found no significant weight loss by imipridone derivatives (Supplementary Figure 8I). With respect to morphological appearance, we noted that tumors treated with imipridones were smaller and on the histopathological level ONC212 showed the most prominent reduction in cellular density and mitotic rate and an increase in cell death (Supplementary Figure 9C and 9E). We assessed the effects on imipridones on cellular proliferation *in vivo* by staining for Ki67. In comparison to vehicle, both ONC201 and ONC212 reduced the proliferation index and number of positive cells (Supplementary Figure 9C). However, ONC212 was most potent. To assess the amount of apoptosis induced by imipridones *in vivo* we stained tumor section for TUNEL. While vehicle and ONC201 revealed minimal staining, ONC212 displayed significantly more positive tumor cells (Supplementary Figure 9D). These observations are in keeping with the significantly smaller tumor size in animals that were treated with ONC212. Taken together, these results intimate

that imipridone derivatives are potential drug compounds for the treatment of malignant glial brain tumors.

Based on these results, we extended our studies to orthotopic patient-derived xenografts of glioblastoma with high or low c-myc expression (Supplementary Figure 8J) in order to test the hypothesis that high levels of c-myc protein predicts a better response to imipridone treatment in the most preclinical relevant models. GBM123 cells (high c-myc protein) were more susceptible to imipridones as compared to U87 GBM cells (low to intermediate c-myc protein levels) (Supplementary Figure 8J-L). For these experiments, we used the most potent imipridone, ONC212, given the well-known challenges of treating orthotopic glioblastoma models. U87 or GBM123 cells were injected into the brain of nude mice. After establishment of tumors, animals were treated with vehicle or ONC212. While in the U87 model we only detected a small survival benefit, we found a substantial increase of overall survival in the ONC212 treatment group in the GBM123 model (Figure 6A and 6B). Notably, when comparing the survival curves of ONC212 in both the U87 and GBM123 model, we found that ONC212 prolonged host survival significantly longer as compared to the U87 model, suggesting that high c-myc protein levels predict a longer host survival upon imipridone treatment ($p < 0.05$).

Given the role of c-myc in imipridone mediated cell death, the *in vivo* implication of high c-myc levels and our earlier findings that interference with c-myc by BRD4 inhibitors enhance the efficacy of imipridones, we assessed the combination treatment strategy of imipridones and BRD4 inhibitors in a xenograft model, utilizing the NCH644, stem-like GBM cells. While single treated host animals revealed a slight reduction in tumor growth, the combined treatment of ONC201 along with OTX015 led to a significant reduction in tumor growth with significant cell death/necrosis (Figure 6C-F). Despite this efficacy, the combination treatment had no effect on the viability of organs, such as brain, heart, lung, liver, intestine, spleen and kidney, and did not induce significant weight loss (Supplementary Figure 9A and 9B). Finally, we confirmed that PHGDH inhibitors and imipridones reduce tumor growth synergistically *in vivo*. To this purpose, we tested the glioblastoma model system, U87-EGFRvIII. Host animals treated with the combination treatment of ONC212 and NCT-503 had significantly smaller tumors than single or vehicle treatments (Figure 6G). As an additional model system, the HCT116 colon carcinoma xenograft model was tested. In like manner, host animals that received the combination treatment of ONC212 and NCT-503 had significantly smaller tumors than single or vehicle treatments (Figure 6H-I and Supplementary Figure 7E-F).

Discussion

Glioblastoma remains one of the biggest challenges in medicine and thus far, researchers have not been successful to provide a substantial improvement in prognosis for this detrimental disease (30). Despite the fact that unlike other tumors GBMs have known recurrent genetic alterations and certain point mutations for which an arsenal of inhibitors already exists, none of these compounds has shown a durable response clinically (31,32). The reason for these failures might be multiple, but certainly related to the fact that GBMs have a substantial, intrinsic heterogeneity that in itself requires either pleiotropic drug

compounds that interfere with multiple pathways or a rational drug combination therapy at the onset (27,33–38).

Imipridone derivatives elegantly fit into this category since these compounds act on several pivotal pathways for gliomagenesis, maintenance and progression of these neoplasms, e.g. AKT and ERK signaling and the anti-apoptotic Bcl-2 family members of proteins (13). The first compound out of this category is TIC10/ONC201 that was discovered in an attempt to identify compounds that increase expression for the death ligand TRAIL (3). Shortly thereafter, it was suggested that ONC201 elicits the induction of an integrated stress response, involving the transcription factor, ATF4 (4), leading to an inhibition of proliferation of cancer cells. However, the underlying mechanism as to how this happens remained elusive and our current study provides significant additional novel insight about the underlying events.

Having discovered a potent class of molecules with anti-cancer properties, it was tempting to develop derivatives that are more potent than the lead compound. Examples of this strategy are the imipridones, ONC206 and ONC212 (2). In our studies, we found that ONC212 was the most potent inhibitor with respect to anti-glioma activity, followed by ONC206 and ONC201. These results are in keeping with one earlier study that focused on pancreatic adenocarcinoma model systems (2).

In seeking more upstream mechanisms as to how imipridones elicit their anti-glioma activity we conducted transcriptome analysis with subsequent GSEA and metabolite analysis by LC/MS. We made the novel observation that imipridones affected energy metabolism by suppression of genes encoding for enzymes and metabolites related to glycolysis and oxidative phosphorylation. Consistently, the proteins related to those genes were downregulated as well. To the best of our knowledge, these findings have not been reported thus far and mechanistically significantly extend the understanding of how imipridones elicit their anti-tumor effects. Given that many tumors are “glycolytic” in nature, it is a notable finding that imipridones interfere with both OXPHOS and glycolysis since many compounds that target only one of these pathways, such as 2-DG, will result in the compensatory activation of other major metabolic energy pathways. For instance, 2-DG inhibits glycolysis and in turn tumor cells activate oxidative phosphorylation to balance their energy needs. Mechanistically, this is owed in part due to mitochondrial plasticity since 2-DG inhibits mitochondrial fission, leading to enhanced fusion by modulation of the phosphorylation status of Drp1 (39). Our findings also show that imipridones regulate mitochondrial plasticity since they lead to enhanced mitochondrial fission, which in turn dampens OXPHOS activity. Another example is gemcitabine resistance in model systems of pancreatic cancer since these tumors enhance glucose metabolism to escape from therapy (40). In this context, it may be tempting to speculate as to whether or not imipridones might reverse gemcitabine resistance or act synergistically to reduce tumor cell proliferation with this chemotherapeutic drug.

To compensate for this massive impact on tumor cell energy metabolism by imipridones, we found that these compounds activate the serine-one carbon-glycine pathway that is utilized by several types of tumors to support growth. Interestingly, the literature also suggest a role

for this pathway to synthesize ATP independent of OXPHOS and glycolysis (41). Therefore, imipridone might utilize this pathway in part to produce energy. In keeping with its pro-survival function (42–45), interference with this pathway through PHGDH inhibition (28), using two novel pharmaceutical inhibitors, leads to synergistic cell death *in vitro* and *in vivo*. Although we have not evaluated methotrexate in combination with imipridone, it is likely that this compound might synergize with imipridones as well since methotrexate interferes with the folate cycle and thus with the serine-glycine pathway.

We determined the mechanism how imipridones suspend energy metabolism in tumor cells and found that the transcription factor c-myc (46–48) is involved in this process. Although c-myc was not downregulated at the level of transcription, we found a marked decrease of c-myc on the protein level as early as 1h after treatment. Consistently, cells with high-levels of c-myc are more susceptible to imipridone treatment, an important finding that has not been shown previously before. Given that GBM stem-like cells are known to be highly dependent on c-myc, our observation that imipridones induce massive apoptosis in these cells is in keeping with this earlier notion. Therefore, our findings suggest that imipridones have the potential to eradicate the stem-cell fraction within a glial neoplasm, thus counteracting the development of treatment resistance and recurrence. Similarly, the pediatric GBM cell line SF188 (c-myc amplified) displays high sensitivity to imipridone derivative, suggesting that c-myc status may predict apoptotic susceptibility to imipridone derivatives. However, it should be noted that even tumor cells with lower expression levels of c-myc display an anti-proliferative response to imipridones, albeit without significant apoptosis induction. Our findings also demonstrate that inhibition of c-myc super enhancers by BRD4 inhibitors along with imipridones synergistically reduce the viability of glioma cells *in vitro* and *in vivo*, which has not been shown in other tumor entities before. Therefore, it might be tempting to speculate whether or not non-solid malignancies might respond to this novel combination therapy as well.

Finally, we confirmed whether or not imipridones prolong host survival in orthotopic xenograft models. Our findings showed that ONC212 extended host survival in a high c-myc expressing patient-derived xenograft model, whereas in a low c-myc expressing model system these anti-glioma effects were significantly less pronounced. These findings support the overall notion that high c-myc levels predict therapeutic responses to imipridones and that chemically modification of imipridones potentially enhance anti-tumor activity.

Taken together, these findings provide a foundation for clinical testing of these compounds either singly or in combination regimes in patients. In addition, utilization of c-myc as a biomarker might be a strategy for prediction of therapeutic responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational relevance statement

Imipridones have recently entered clinical testing for hematological and solid malignancies, including glioblastoma, the most common primary brain tumor that requires novel treatments. In the current study, we provide evidence that chemically modified imipridones, ONC206 and ONC212, exert more potent anti-glioma activity than the original lead compound, ONC201. Moreover, we demonstrate that high levels of c-myc predict therapeutic responses in preclinical model systems of glioblastoma. Notably, single treatment of ONC212 significantly extends host survival in an orthotopic patient-derived glioblastoma xenograft model. Combined treatment with a clinically validated BRD4 antagonist, OTX015, and imipridones induces enhanced reduction of glioma growth in vitro and in vivo. These observations support the notion that chemically modified imipridones display preclinical activity singly or as part of combination therapies in model systems of glioblastoma. Therefore, clinical testing of these novel compounds either singly or in combination therapies is warranted.

Figure 1: Imipridones cause inhibition of cellular proliferation in a range of model systems of glioblastoma, including patient-derived xenograft (PDX), stem-like and established GBM cells, and induce apoptosis in a c-myc protein expression level dependent manner.

A, Chemical structures of the imipridones used in the current study. The lead compound is ONC201, while ONC206 and ONC212 are chemical derivatives. B, High c-myc expressing GBM cultures, NCH421k (stem-like GBM cells), NCH644, NCH690 and pediatric SF188 GBM cells were treated with increasing concentrations of ONC201, ONC206 and ONC212 for 72h, fixed, stained with propidium iodide and analyzed by flow cytometry. Shown are mean percentages with SD of cells in the sub-G1 fraction. C, low c-myc expressing established GBM cells, LN229, U87, T98G and PDX cells, GBM14, were treated with increasing concentrations of ONC201, ONC206 and ONC212 for 72h were fixed, stained with propidium iodide and analyzed by flow cytometry. Shown are mean percentages with SD of cells in the sub-G1 fraction. D, GBM stem-like cells, NCH421k, NCH644, NCH690, established GBM cells, SF188 (c-myc amplified, pediatric GBM), LN229, U87, T98G and GBM14 (patient derived xenograft GBM cells) were analyzed by capillary electrophoresis

for the protein expression levels of c-myc and Actin. Two stars (**) and one star (*) indicate long and short exposure times, respectively. E, High (NCH421k), intermediate (U87) and low (GBM14) cells were analyzed for the expression of c-myc. D-E constitute same samples with different exposures. F, Cell cultures analyzed in panel E were treated with ONC201 at 10 μ M for 72h and analyzed for the percentage of apoptotic cells by flow cytometry (data extrapolated from the analysis in panel 1B and 1C). Cells were then grouped in accordance with their c-myc levels (high: NCH421k, NCH644, NCH690 and SF188; intermediate to low: LN229, U87, T98G and GBM14). Shown are mean values with SD ($p < 0.05$). G, Cells from panel E (high c-myc: NCH421k, intermediate c-myc: U87 and low c-myc: GBM14) as well as non-neoplastic human astrocytes were treated with increasing concentrations of imipridones. Non-linear-regression was used to calculate the IC_{80} values. H, I U87 GBM cells were transduced with a control (empty) or human c-myc encoding lentivirus and selected by puromycin. Enhanced expression of c-myc was verified by capillary electrophoresis (I). Control and c-myc overexpressing U87 cells were treated with 10 μ M ONC201, fixed and stained with propidium iodide and analyzed for DNA fragmentation by flow cytometry J, K, SF188 (pediatric glioblastoma with *MYC* amplified) were transduced with lentiviral particles, containing either a non-targeting (NT) or human c-myc targeting shRNA. Cells were selected with puromycin to obtain stable cultures. Silencing of c-myc was confirmed by capillary electrophoresis (K). Non-targeting and c-myc shRNA containing cells were treated with 10 μ M ONC201 for the indicated time frame, fixed, stained with Propidium iodide and analyzed for DNA fragmentation. Shown are means with SD.

Figure 2: Imipridones suppress protein levels of the transcription factor, c-myc, through modulation of its stability via GSK3 β .

A, B, U87 were treated with the indicated imipridones derivatives. After the indicated time points, whole-cell protein lysates were prepared and subjected to capillary electrophoresis, using the Wes instrument (Proteinsimple, CA). C, U87 GBM cells were treated with DMSO or ONC201, ONC206 or ONC212 at the indicated concentrations for 72h. Thereafter, RNA was isolated, subjected to microarray analysis, followed by gene set enrichment analysis (GSEA). Shown is the gene set for MYC related transcriptional targets. NES: Normalized enriched score. D, U87 GBM cells were treated with the indicated imipridones. Whole cell protein lysates were harvested and analyzed by capillary electrophoresis for c-myc and Vinculin. E, U87 GBM cells were pre-treated with ONC212. Thereafter, cells were exposed to cycloheximide for the indicated time points. Whole-protein lysates were collected and analyzed for the expression of c-myc by capillary electrophoresis. F, Quantification for the analysis as shown in E. Shown are means of relative percentages compared to either vehicle

or ONC212 treated samples (timepoint 0h). G, U87 GBM cells were treated with ONC212 in the presence or absence of MG132. Whole protein lysates were collected and analyzed by capillary electrophoresis. H, U87 GBM cells were treated as indicated and whole protein lysates were prepared. Lysates were subjected to capillary electrophoresis on the Wes simple instrument (Proteinsimple, CA). Shown are protein levels of phosphorylated Akt (Serine 473), total Akt, phosphorylated GSK3 β (Serine 9), total GSK3 β , phosphorylated c-myc (Threonine 58), total c-myc and Vinculin. I, U87 GBM cells were treated in the presence or absence of GSK3 β inhibitors, CHIR-908014 or SB216763. Whole cell protein lysates were harvested and analyzed for the expression of c-myc and Vinculin. J, U87 GBM cells were transfected with a plasmid for c-myc wild-type or c-myc T58A mutant (both containing a His-tag). Thereafter, GBM cells were treated with vehicle or ONC201 at the indicated concentration. Whole cell protein lysates were collected and subjected to standard western blotting analysis. Shown are the expression levels for His and Actin.

Figure 3: Imipridones, ONC201, ONC206 and ONC212, suppress glycolytic energy metabolism.

A, U87 GBM cells were treated with ONC201 or ONC212 for 72h. Thereafter, RNA was analyzed and subsequently whole-transcriptome analysis was performed by microarray analysis, followed by gene set enrichment analysis (GSEA). Shown are plots for glucose starvation (up-regulated), glycolysis (down-regulated) and oxidative phosphorylation (down-regulated). NES: normalized enrichment score. B, U87 GBM cells were treated with ONC201 and ONC212 for 24h and analyzed for ATP content. Shown are mean values with SD. n=3 biological replicates. * indicates a p<0.05 between control and treatment. C, D, SF188 (C) U87 (D) GBM cells were treated with the indicated compounds for 24h. Thereafter, extracellular flux analysis for ECAR (extracellular acidification rate) was performed in the context of a glycolytic – stress assay. Shown are ECAR values. Shown are means with SEM. G: Glucose, OM: Oligomycin, 2-DG: 2-deoxyglucose. E, Comparison between ECAR values in SF188 (high c-myc) and U87 GBM (intermediate c-myc) cells. F, Empty vector or c-myc transduced U87 cells were treated with ONC212 and analyzed for

ECAR. Shown are percentages relative to the controls. G, U87 GBM cells were treated with the respective imipridones as indicated for 72h and analyzed for the expression of glycolytic transporters (GLUT1) and enzymes (LDHA, HK2). H, GBM cells were transfected with two different siRNA oligonucleotides, targeting c-myc, or with non-targeting siRNA. 72h after transfection, whole protein lysates were prepared and analyzed by capillary electrophoresis for glycolytic transporters (GLUT1) and enzymes (HK2, LDHA) as well as for c-myc expression. I, U87 GBM cells were treated with 5 μ M OTX-015 (an inhibitor of BRD proteins). Cells were analyzed by extracellular flux analysis in the context of a glycolytic stress assay. Shown are means and SD. ** means p-value less than 0.01, whereas * indicates p of less than 0.05. J, U87 GBM cells were treated with vehicle or ONC212. Thereafter, cells were harvested for LC/MS analysis for the indicated glycolytic or pentose phosphate pathway related metabolites. Glu: Glucose, G-6P: Glucose-6-phosphate, F1,6BP: Fructose-1,6-bisphosphate, 3PGA: Glyceraldehyde-3-phosphate, 3-PG: 3-Phosphoglycerate, PEP: Phosphoenolpyruvate, Pyr: Pyruvate, DHAC: Dihydroxyacetonephosphate. 6-PG: 6-Phosphogluconate, RR-5-P: Ribose-ribulose-5-phosphate.

Figure 4: Imipridones inhibit oxidative phosphorylation, reduce the expression of respiratory complexes, metabolites of the TCA cycle and mitochondrial biogenesis.

A, B, U87 GBM cells were treated as indicated for 24h and analyzed for extracellular flux (Seahorse XFe24). Shown are mitochondrial respiration (mitochondrial OCR) and OXPHOS related ATP production. Shown are means and SD. C, SF188 and U87 GBM cells were treated as indicated and subjected to assessment of the OCR. Shown are percentages relative to control. D, U87 GBM cells were treated as indicated for 72h. Whole cell protein lysates were collected and analyzed by conventional western blotting. Shown are the expression of the five respiratory complexes and pyruvate dehydrogenase (PDH). E, GBM cells were transfected with one siRNA oligonucleotide, targeting c-myc, or with non-targeting siRNA. 72h after transfection, whole protein lysates were prepared and analyzed for the expression of respiratory complexes and pyruvate dehydrogenase (PDHE) (conventional western blotting). F, U87 GBM cells were transduced with non-targeting or MYC specific shRNA lentiviral particles. Stable c-myc and non-targeting shRNA expressing cells were selected.

Thereafter, whole cell protein lysates were prepared and analyzed for the expression of respiratory complexes and pyruvate dehydrogenase (PDHE) (conventional western blotting). G, U87 GBM cells were treated as indicated for 24h and subjected to extracellular flux analysis. Oxygen consumption rate (OCR) was determined. Shown are means with SD. H, U87 GBM cells were treated with vehicle, ONC212 or ONC201. Thereafter, cells were harvested for LC/MS analysis for metabolites related to the TCA cycle. I, U87 GBM cells were treated with imirpidone derivatives as indicated, RNA was harvested and cDNA was analyzed for mtDNA levels relative to nuclear levels. Shown are means with SD. ** means p-value less than 0.01, whereas * indicates p of less than 0.05.

Figure 5: Imipridones activate the serine-one carbon-glycine pathway and interference with this pathway sensitizes for imipridone mediated apoptosis in vitro and in vivo

A, U87 GBM cells were treated with DMSO or ONC201, ONC206 or ONC212 at the indicated concentrations for 72h. Thereafter, RNA was isolated, subjected to microarray analysis, followed by gene set enrichment analysis (GSEA). Shown is the gene set for serine-one carbon-glycine related transcriptional targets. NES: Normalized enriched score. B, U87 GBM cells were treated as indicated. RNA was harvested, reverse transcribed and cDNA was amplified for PHGDH, PSAT1 and PSPH. Shown are means and SD. C, U87 GBM cells were treated with imipridones. Lysates were processed for LC/MS and analyzed for the indicated metabolites related to serine-one carbon-glycine metabolism. IMP: inosine monophosphate. D, U87 GBM cells were treated with the indicated imipridones. Whole cell protein lysates were prepared and analyzed for expression of the indicated proteins. E, U87 GBM cells were transfected with non-targeting siRNA or ATF4 specific siRNA. Thereafter, cells were treated with the indicated imipridone. Whole cell protein lysates were harvested and analyzed for the indicated proteins by capillary electrophoresis. U87 cells were

transduced with non-targeting shRNA or ATF4 specific lentiviral particles. Stable cell lines were generated, plated and treated with ONC212. Whole cell protein lysates were collected and analyzed for the expression of the indicated proteins (right panel). F, G GBM stem cells, NCH421k, NCH644 and NCH690, and established GBM cells, U87, were treated with imirpidones in the presence or absence of PHGDH inhibitor, NCT-503 or CBR-5884 for 96h. Thereafter, cells were labeled with annexin V/propidium iodide and analyzed by flow cytometry. Shown are means and SD. **/**/**/**/** means p-value less than 0.01, whereas * indicates p of less than 0.05.

Figure 6: Imipridones extend host survival in patient-derived xenograft (PDX) models of GBM and reduce tumor growth synergistically with BRD4 and PHGDH inhibitors

A, U87 GBM cells (low to intermediate c-myc levels) were implanted intracranially in nude mice. After randomization in two groups, treatment was initiated with either vehicle or ONC212 (100 mg/kg) twice a week. Primary endpoint for these experiments is survival or a moribund state of the animal. Shown are Kaplan-Meier-survival curves and the log-rank test was applied to calculate statistical significance. B, Patient-derived xenograft cells, GBM123 (high c-myc expressing tumors), were implanted intracranially in nude mice. After randomization in two groups, treatment was initiated with either vehicle or ONC212 (100 mg/kg) twice a week. Primary endpoint for these experiments is survival or a moribund state of the animal. Shown are Kaplan-Meier-survival curves and the log-rank test was applied to calculate statistical significance. C, D, E, Stem-like GBM cells, NCH644, were implanted subcutaneously. Once tumors were established, animals were randomized into four designated groups, vehicle, ONC201 (100 mg/kg), OTX015 (OTX) (75 mg/kg) or the

combination of ONC201+OTX015. Treatments were given three times a week. Caliper measurements were performed and tumor volumes were calculated as previously described. At the end of the experiment, statistical analysis was performed, using the Mann-Whitney test. A p value of less than 0.05 was considered as statistical significant. Shown are scatter plots with means and SD. Gross images of the explanted tumors are provided in panel E. F, Representative histological images of the individual treatments of the xenografts performed in C. Slides were stained with hematoxylin and eosin (HE). G, U87-EGFR-vIII cells were implanted subcutaneously in nude mice. After establishment of tumors, four groups were formed as indicated: Vehicle, ONC212 (50 mg/kg), NCT-503 (50 mg/kg) or the combination of ONC212 and NCT-503. Shown are scatter plots of relative tumor fold changes in (%) with means and SD (last day of experiment; normalization is to 100% at the start of the experiment). H, HCT116 cells were implanted subcutaneously in nude mice. After establishment of tumors, four groups were formed as indicated: Vehicle, ONC212 (50 mg/kg), NCT-503 (50 mg/kg) or the combination of ONC212 and NCT-503. Shown are means and SD. I, Scatter plots of the final tumor sizes on the day of conclusion of the experiment. ** means p-value less than 0.01, whereas * indicates p of less than 0.05.