



Original Articles

IP1867B suppresses the insulin-like growth factor 1 receptor (IGF1R) ablating epidermal growth factor receptor inhibitor resistance in adult high grade gliomas



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ABSTRACT

High grade gliomas (HGGs) are aggressive primary brain tumours with local invasive growth and poor clinical prognosis. Clinical outcome is compounded by resistance to standard and novel therapeutics. We have evaluated reformulated aspirin (IP1867B) alone and in combination with conventional and novel anti-aHGG agents. We show that recent biopsy-derived aHGG models were highly resistant to conventional therapeutics although show sensitivity to IP1867B, a reformulated “liquid” aspirin. IP1867B treatment mediated a potent suppression of the IL6/STAT3 and NF-κB pathways and observed a significant reduction in EGFR transcription and protein expression. We observed the loss of the insulin-like growth factor 1 and insulin-like growth factor 1 receptor expression at both the transcript and protein level post IP1867B treatment. This increased sensitivity to EGFR inhibitors. In vivo, IP1867B was very well tolerated, had little-to-no gastric lesions versus aspirin and, directed a significant reduction of tumour burden with suppression of EGFR, IGF1 and IGFR1. With EGFR inhibitors, we noted a potent synergistic response in aHGG cells. These data provide a rationale for further investigation of IP1867B with a number of anti-EGFR agents currently being evaluated in the clinic.

1. Introduction

In 2017, 80,000 patients in the US and 11,400 in the UK were diagnosed with a primary brain tumour [1,2]. Paediatric high grade glioma frequency in the US are 5.7 cases per 100,000. Adult HGG treatment comprises surgical resection and combined radio- and chemo-therapy [3,4]. The standard chemotherapeutic is temozolomide (Temodal < SUP > TM < /SUP >) (TMZ), an oral DNA alkylating agent [5–7]. TMZ and radiotherapy (the “Stupp protocol”) demonstrated significant patient survival benefit versus radiotherapy alone, showing a median increase in survival of 2.5 months [3,4,8]. Cohort analysis revealed a discernible clinical response only in adult high grade glioma (aHGG) patients exhibiting promoter methylation of *O6-methylguanine DNA methyltransferase (MGMT)* [9]. Irrespective of *MGMT* promoter methylation, almost all aHGG patients exhibited eventual disease progression. An area that has attracted attention is the repurposing of Food and Drug Administration (FDA) approved agents for difficult-to-treat cancers, in particular high grade gliomas [10–12].

Aspirin (acetylsalicylic acid, ASA) is a nonsteroidal anti-inflammatory drug which inhibits the cyclooxygenase (COX) enzymes COX-1 and COX-2 [13] and has been implicated in anti-cancer responses [14]. Aspirin use post-diagnosis improved patient outcome, suggesting a role with conventional therapies [15]. The long-term use of aspirin reduced the cancer risk in paediatric patients with constitutional mismatch repair deficiency who are predisposed to cancer development [16]. There are over 20 registered clinical trials of aspirin for cancer therapy [17]. Aspirin solubility is low (1 g in: 300 mL water at 25 < SUP > O < /SUP > C) and there are serious concerns regarding gastrointestinal (GI) injury in patients prescribed aspirin long-term. Even low dose aspirin can induce duodenal mucosal injury including ulcers or haemorrhages.

Approximately 50% of aHGG display *EGFR* amplification. Further, a number of aHGGs express a truncated EGFR (EGFRvIII) protein which is generated following the removal of exons 2–7 [18,19] that displays constitutive, ligand-independent tyrosine kinase activity [20]. Insulin Like Growth Factor 1 Receptor (IGF1R) is rarely mutated or amplified

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in aHGG [21], where, activation of this network is considered ligand-dependant via endocrine mechanisms. IGF1R targeting has a significant impact on AKT signalling in aHGG [22], and is implicated in EGFR inhibitor resistance [23].

We report that IP1867B (ASA/triacetin/saccharin) directed a potent anti-aHGG response *in vitro* and *in vivo*. This was via suppression of the IL6/STAT3, NF- κ B, IGF1/IGFR1 signalling networks. IP1867B exposure induced the down regulation of EGFR, at both protein and transcript level. IP1867B was well tolerated in non-neoplastic astrocytes and showed no associated *in vivo* GI toxicity. IP1867B synergised with EGFR inhibitors and in combination with IGF1R inhibitors had no additive effect.

2. Materials and methods

2.1. Tumour specimens and primary tumour cultures

Adult HGG biopsy samples were obtained from patients undergoing biopsy surgery at Kings College Hospital NHS Foundation Trust (LREC review board (11/SC/0048) London, UK). Tumours were classified based on WHO criteria [1] after examination by neuro-pathologists. Tumour mass was mechanically dissociated into explant clumps, incubated at 37 °C to allow neoplastic cells to colonize the flask/cell culture plates. Medium was changed every 2 days. When neoplastic cells reached confluence, cells were passaged and expanded. Once passage 1 cells were obtained, Short Tandem Repeat (STR) analysis was conducted to enable subsequent cell line authentication (Agilent Bioscience).

2.2. Chemotherapeutics and cell culture

IP1867B (Innovate Pharmaceuticals), TMZ (T2577), vincristine (V0400000) and aspirin (Sigma-Aldrich). Gemcitabine (S1714), Gefitinib or AZD3759 (SelleckChem). Adult glioma cell line U87MG was obtained from the ATCC and maintained in DMEM supplemented with 10% heat inactivated FCS (Sigma-Aldrich). Novel aHGG biopsies UP-029, SEBTA-003, SEBTA-023 and SEBTA-025 were cultured in DMEM medium. CC2565 non-neoplastic astrocytes (Lonza) were maintained in astrocyte growth medium supplemented with SingleQuots™ (CC-3187 Lonza) including (CC-4123): rhEGF, insulin, ascorbic acid, L-glutamine. Cells were cultured under normoxic (21%) or hypoxic (1% O₂) conditions.

2.3. MTS cell viability assay

5 × 10³ cells were seeded in triplicate in a 96 well plate. 24 h post seeding, cell lines were treated with each agent. CellTiter 96® Aqueous One Solution MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Reagent (G3580 Promega) was added and absorbance at 490 nm recorded.

2.4. Western blot analysis

Cells were harvested and lysed using RIPA buffer (89900, Thermo-Fisher) and 1x protease inhibitor cocktail (78442 Thermo-Fisher). 50 µg total protein was loaded per sample and separated by SDS-PAGE. Proteins were transferred onto PDVF membrane (BioRad). Membranes were blocked for 1 h at room temperature (RT) in Odyssey blocking buffer (972–46100 LICOR). Primary antibodies (Supplemental Table 1) were added overnight at 4 °C. Secondary antibody were added (LICOR) at 1:10000 dilution for 1 h at RT and membranes imaged on an Odyssey CLX (Licor). All uncropped immunoblots are shown in Supplemental Figs. 1–5.

2.5. Quantitative real time PCR

Total RNA was extracted (RNAeasy, 74104 Qiagen) and measured using RNA6000 chip (Agilent Bioscience, UK). 0.5 µg total RNA was used per cDNA synthesis reaction (iScript cDNA synthesis kit, 1708890 BioRad). Real Time PCR was performed using a LightCycler 96 (Roche) (iTaq SYBR Green, 1725120 BioRad). Primer sequences shown in Supplemental Table 1. Data analysis was carried out using the 2^{- $\Delta\Delta$ CT} method [24].

2.6. RT² pathway transcriptomics and analysis

Total RNA was extracted per condition (NT, 6hr IP1867B) using the RNeasy Plus Mini Kit (Qiagen) including genomic DNA removal. The quantified by RNA 6000 Nano Kit (Agilent, UK). 0.5 µg of RNA was used for RT² cDNA (Qiagen, UK). cDNA was added on the cell death Pathway Finder (PAHS-212Z), NF- κ B signalling array (Qiagen, PAHS-025Z) and IL6/STAT3 signalling pathway (PAHS-160Z). Plates were run using a Light Cycler 96 (Roche, UK). The CT values were obtained and fold changes determined by 2^{- $\Delta\Delta$ CT} [24].

2.7. In vivo anti-tumour efficacy

250,000 U87-luciferase cells were inoculated (intra-cranially) into NOD/SCID mice (aged 10–12 weeks). Animals were grouped based on luciferase imaging. Treatment was initiated every three days via IP injection, PBS, TMZ 50 mg/kg/day or IP1867B 30 mg/kg/day. *In vivo* bioluminescent imaging was performed once per week and all animals were weighed with behaviour and neurological signs (including altered gait, tremors, seizures and/or lethargy) monitored daily. This animal study was conducted with Institutional and Home Office ethical approval.

2.8. In vivo gastric studies

Female C57BL/6 mice (aged 10–12 weeks, n = 2–3 mice/group) were fasted for 16 h and oral gavaged daily for 5 consecutive days with 1% methylcellulose (10 mL/kg), IP1867B (30 mg/kg) or aspirin (30 mg/kg). All animal studies were conducted with Home Office ethical approval. Stomachs were extracted and opened along their greater curvature and pinned to a bed of paraffin and formaldehyde fixed. Stomachs were cut into strips containing antrum and corpus and processed for standard H&E staining. Non-serial sectioned gastric mucosae were assessed for lesion number (521 ± 112 mm/stomach). All animal studies were conducted with Institutional and Home Office ethical approval.

2.9. Data analysis and statistics

Studies were analysed using GraphPad Prism and are represented as mean ± St.Dev. Statistical significance calculated using Student's t-test, (*P ≤ 0.05), two-tailed ANOVA analysis, or the log-rank test for Kaplan Meier survival analyses.

3. Results

3.1. IP1867B demonstrates a potent in vitro anti-aHGG effect under both normoxic and hypoxic conditions

We investigated how novel biopsy-derived aHGG responded to each excipient, IP1867B and temozolomide (TMZ) (Fig. 1). 96 h post drug treatment MTS viability assays were conducted and EC₅₀ values determined (Fig. 1A–D). Saccharin did not affect cell viability at even the highest tested concentration of 36.5 mM. Triacetin significantly reduced cell viability although this was only at dosages > 290 mM. When treated with aspirin alone, aHGG cells show limited cell death. The

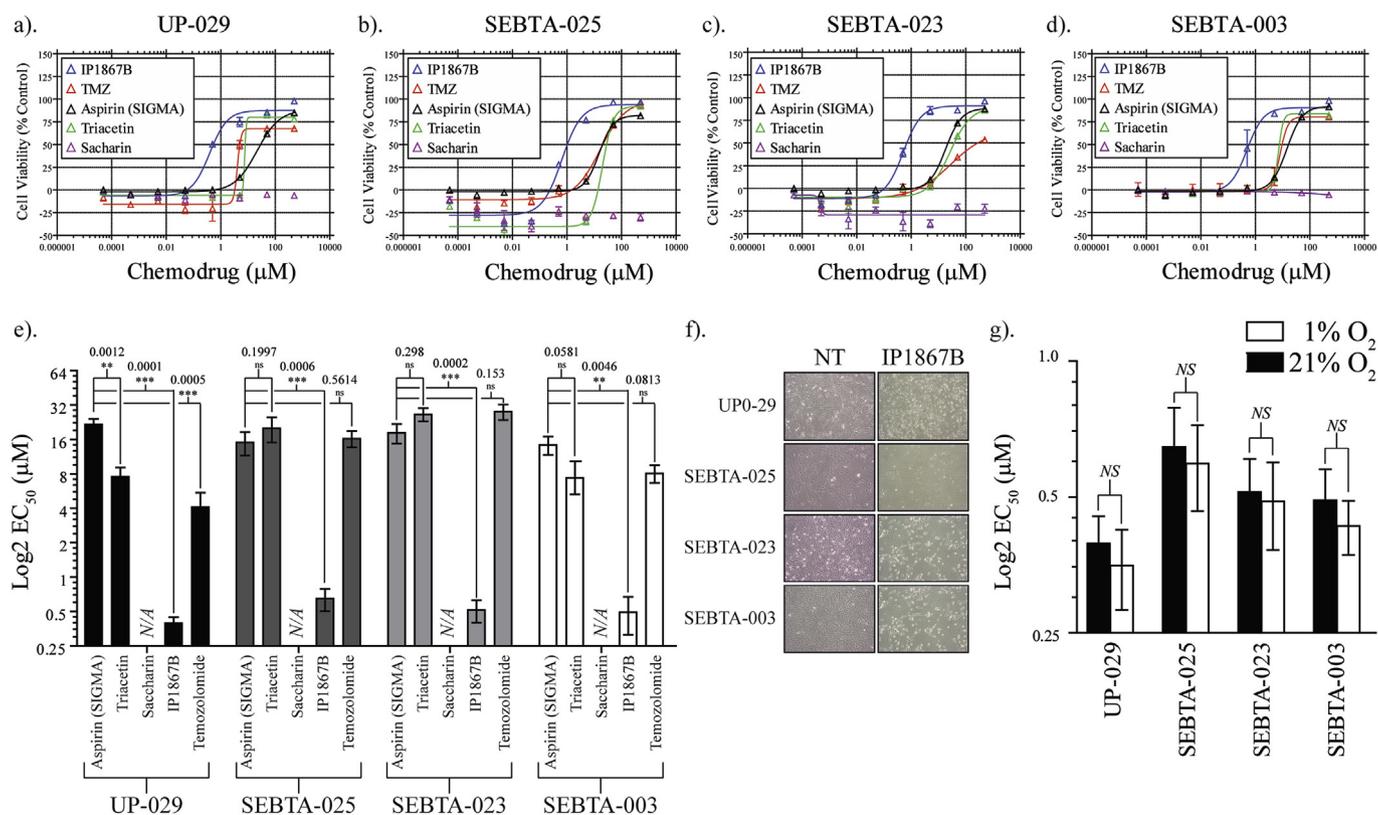


Fig. 1. IP1867B treatment of biopsy-derived aHGG cells significantly reduces cell viability. a-d). Indicated aHGG cells (10,000 per well) were treated with varying dosages of each indicated compound and 96 h post treatment, MTS (Promega) assays were conducted. $n = 3$, error bars indicate \pm StDev. e). Average Log_2 EC_{50} concentration for each excipient shown. 2-tailed ANOVA analysis indicated by each bracket with P value and significance shown. $n = 3 \pm$ StDev. f). Representative microscopy images for each biopsy-derived aHGG cell line 24 h post IP1867B treatment (40 \times magnification). g). Average Log_2 EC_{50} concentration 96 h post IP1867B treatment under normoxic or hypoxic conditions. 2-tailed ANOVA analysis indicated by each bracket with P value and significance shown. $n = 3 \pm$ StDev.

maximal solubility of aspirin (SIGMA Aldrich) was 3 mg/mL (16.65 mM). We were unable to dissolve aspirin at this concentration as significant particulate was observed. The highest concentration used was 1 mg/mL (5.55 mM). Reduced aHGG cell viability was only observed at 500 μM aspirin. We noted a significant reduction in aHGG cell viability following IP1867B treatment. Aspirin alone (SIGMA) and IP1867B noticeably changed the pH of the medium, consistent with high concentration acetylsalicylic acid. This alone did not account for the reduced cell viability as IP1867B significantly reduced aHGG cell viability at lower concentrations (Fig. 1).

We determined the overall EC_{50} for each component and for TMZ (Fig. 1E). Following a single treatment, IP1867B was significantly more potent compared to each agent and single dose TMZ exposure, a response conserved in a number of aHGG cell lines. The aHGG lines demonstrated high tolerance to single dose treatment with TMZ, particularly SEBTA-023, SEBTA-003 and UP-029 that display unmethylated *MGMT* promoters. Representative microscopy images of each aHGG cell line 24 h post IP1867B exposure revealed widespread cell death post-IP1867B treatment that was not observed post-treatment with any of the excipient components with only limited cell detachment/death post-TMZ treatment (Fig. 1F).

Adult HGG therapeutics need to be effective under both normoxic (21% O_2) and hypoxic (1% O_2) conditions. Cells were incubated under hypoxic conditions and treated with IP1867B. At 96 h MTS assays were conducted (under either hypoxic or normoxic conditions) and EC_{50} concentrations determined for each (Fig. 1G). Irrespective of the environment, there was a significant reduction in cell viability post-IP1867B treatment. However, there was no significant difference between the EC_{50} values for each aHGG line cell under normoxic or

hypoxic conditions post-IP1867B exposure.

3.2. IP1867B formulation demonstrates synergy compared to each individual excipient component and increased the effectiveness of TMZ

We questioned if the IP1867B formulation had a synergistic effect on treated aHGG cells. We determined the effective concentration for aspirin (SIGMA) (16 μM) and triacetin (0.6 mM) alone, an effective concentration for aspirin (SIGMA), triacetin and saccharin (the three excipients individually combined at 11 μM , 0.5 mM and 500 mM respectively) and finally for TMZ alone (9.1 μM). Single/dual agents were added simultaneously and were compared to IP1867B (Fig. 2A–D). Treatment with aspirin (SIGMA) and TMZ was additive (where the TMZ EC_{50} fell between 6.1 and 27.2 μM for each aHGG cell line). While there was a reduction in cell viability, this reduction was not statistically significant. When aspirin (SIGMA) and triacetin were used in combination to treat aHGG cells no significant change was noted. A potent synergistic response was observed when IP1867B was compared to each excipient component, or when the three individual components were independently combined (Fig. 2E). When aspirin (SIGMA) and TMZ were used in combination, there was no significant difference in overall aHGG cell viability indicating that the combination of these *in vitro* did not have an additive effect (TMZ EC_{50} remained at 12.1 μM) (Fig. 2F). IP1867B and TMZ when used together, showed an additive effect where the EC_{50} was \approx 0.5 μM (Fig. 2G). We concluded that IP1867B was highly synergistic compared to each excipient component alone and while aspirin (SIGMA) and TMZ in combination did not significantly affect cell viability (either synergistic or additive), IP1867B and TMZ did have an additive response. We questioned if non-neoplastic cells

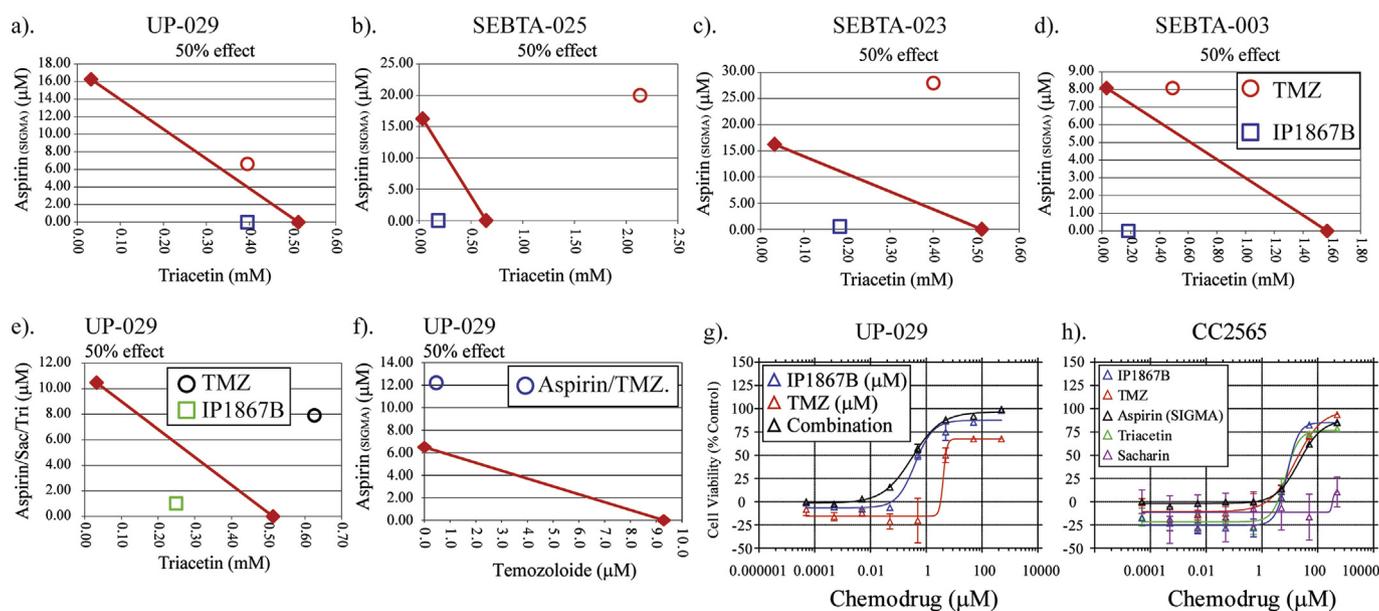


Fig. 2. IP1867B demonstrates synergy compared to each excipient component when patient-derived aHGG cells are treated. a-d). Representative isobolograms from combination or single treatment of aspirin (SIGMA), triacetin, IP1867B or temozolomide in aHGG cell lines. a). UP-029, b). SEBTA-025, c). SEBTA-023, d). SEBTA-003 cells were treated with each effective 50% concentration. IP1867B (or temozolomide) was added to each cell line. EC_{50} values were determined from dose-response curves. The straight line connects the EC_{50} values for each agent alone and illustrates the theoretical values resulting in additive effects. Data points below the line represent synergistic ($CI \leq 0.8$) and above the line antagonistic ($CI \geq 1.2$) interactions. $n = 3$. e-f). Representative isobolograms from combination or single treatment of aHGG cells with IP1867B and TMZ or aspirin and TMZ. $n = 3$ g). Representative isobologram dose curves for UP-029 aHGG cells treated with each indicated agent. h). CC2565 cells (10,000 per well) were treated with varying dosages of each indicated compound and 96 h post treatment, MTS (Promega) assays were conducted. $n = 3$, error bars indicate \pm StDev.

were sensitive to IP1867B. Non-neoplastic CC2565 astrocytes were exposed to each individual component or to IP1867B (Fig. 2H). At 96 h post IP1867B, CC2565 cells did not display a significant reduction in overall viability. IP1867B showed cancer-selectivity (EC_{50} concentration(s) for aHGG was between 0.39 and 0.6458 μ M and the CC2565 EC_{50} was 10.16 μ M) ($P = 0.0038$).

3.3. IP1867B treatment induces the potent suppression of NF- κ B and IGF1R

We examined how IP1867B could mediate this response. We tested if there was significant caspase 3 cleavage following treatment with the IP1867B (Fig. 3A). There was the significant detection of cleaved caspase 3 at 96 h post-treatment. We questioned if there was p53 accumulation and activation. We did not see discernible p53 accumulation although noted p53 phosphorylation (Ser15 and Ser46) (Fig. 3B). In SEBTA-023 aHGG cells we were unable to detect any p53. To complement this study, each aHGG cell line was exposed to IP1867B or TMZ (100 μ M) and at 24 and 96 h post treatment FACS analysis was conducted (Fig. 3C). We detected significant accumulation of sub-G₁ cell population consistent with cell death post IP1867B treatment. At 96 h post-treatment, TMZ exposure also increased the sub-G₁ cell population, however this was significantly lower compared to IP1867B treated aHGG cells.

We evaluated the aHGG transcription response post IP1867B exposure and examined a diverse range of cell death networks, the NF- κ B pathway, and the inflammation response (Fig. 3D, E and F). Three housekeeping genes *Beta-2-microglobulin* (B2M), *hypoxanthine phosphoribosyltransferase 1* (HRPT1) and *Glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) were used for data normalization, and the fold change for each gene of interest was calculated. 12 h post IP1867B treatment, we noted a significant down regulation of various pro-inflammatory genes including *interleukin (IL)12A*, *IL18*, *IL4*, *IL5*, *IL6*, *IL6-receptor*, *tumour necrosis factor (TNF)*, *TNF-receptor 1A* and *TNF-receptor 11B* (Fig. 3G). When treated with IP1867B, aHGG cells directed a significant transcription increase in *IL11*. IL6 drives many of cancer

hallmarks via the downstream activation of STAT3. IL11 has been shown to direct a more prominent role compared to IL6 in inflammation associated cancers, suggesting a potential compensatory mechanism directed by aHGG following IP1867B exposure [25]. These data raise the hypothesis that IP1867B could significantly complement a number of IL11 inhibitors (such as Bazedoxifene and mIL-11 Mutein) currently being tested in various gastrointestinal and breast cancers [25–27].

The related genes *Insulin-like growth factor 1 (IGF1)* and *Insulin-like growth factor 1 receptor (IGF1R)* were identified by our analysis post-IP1867B treatment (Fig. 3G). *IGF1* and *IGF1R* both showed a highly significant expression decrease of 9.42 and 7.39 fold versus untreated aHGG cells respectively. *IGF1* and *IGF1R* can confer chemo-resistance and have been considered important for EGFR inhibitor resistance [28]. These data suggested that IP1867B could significantly inhibit *IGF1* and *IGF1R* by potentially reducing their expression and may, sensitise aHGGs to these therapeutics. Following IP1867B treatment, we noted subtle, although statistically significant, increases in *caspase 3*, *caspase 6* and *caspase 9* expression supporting the induction of apoptosis (Fig. 3G). We observed the direct repression of *EGFR* (2.73 fold reduction) post-IP1867B treatment of UP-029 aHGG cells. These aHGG lines showed significant *EGFR* over expression and suggested a second mechanism where IP1867B could significantly enhance the effectiveness of aHGG therapeutics, in particular EGFR inhibitors supported by *IL4* and *IL6* repression.

We broadened our findings and included additional, recent biopsy-derived aHGG cell lines (Fig. 4A, B and C). IP1867B directed an anti-inflammatory transcription response, characterized by the repression of *IL6*, *IL6-receptor*, *TNF*, *TNF-receptor 1A* and the significant induction of *IL11*. There was a significant reduction of *EGFR*, *IGF1* and *IGF1R* transcription. We questioned if these transcriptional data were conserved at the protein level and noted that there was a considerable reduction of *IGF1R*, *IL6R* and *EGFR* protein expression post-IP1867B treatment in our aHGG cells (Fig. 4D). These data raised the interesting hypothesis that IP1867B treatment could complement EGFR inhibitors.

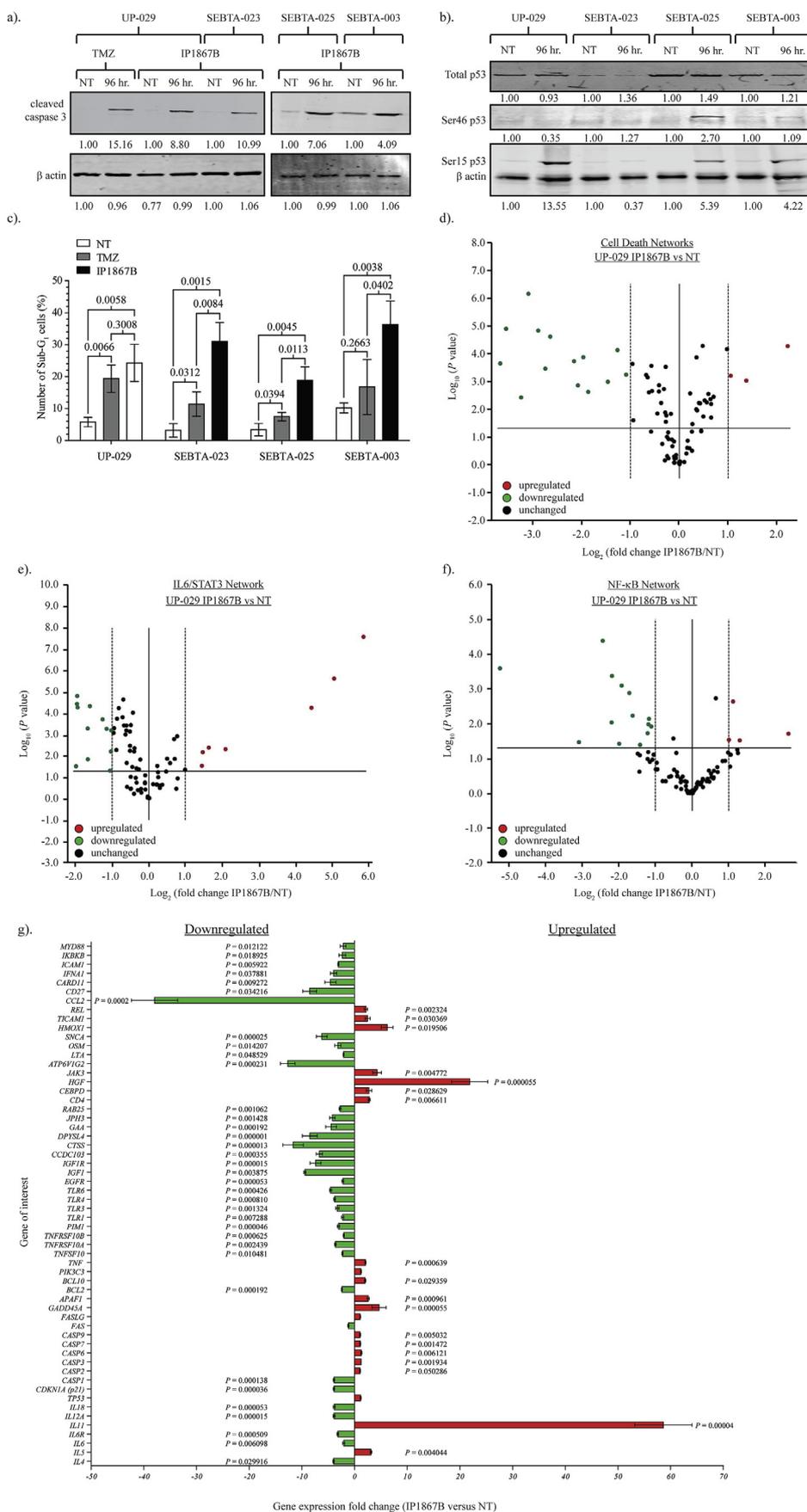
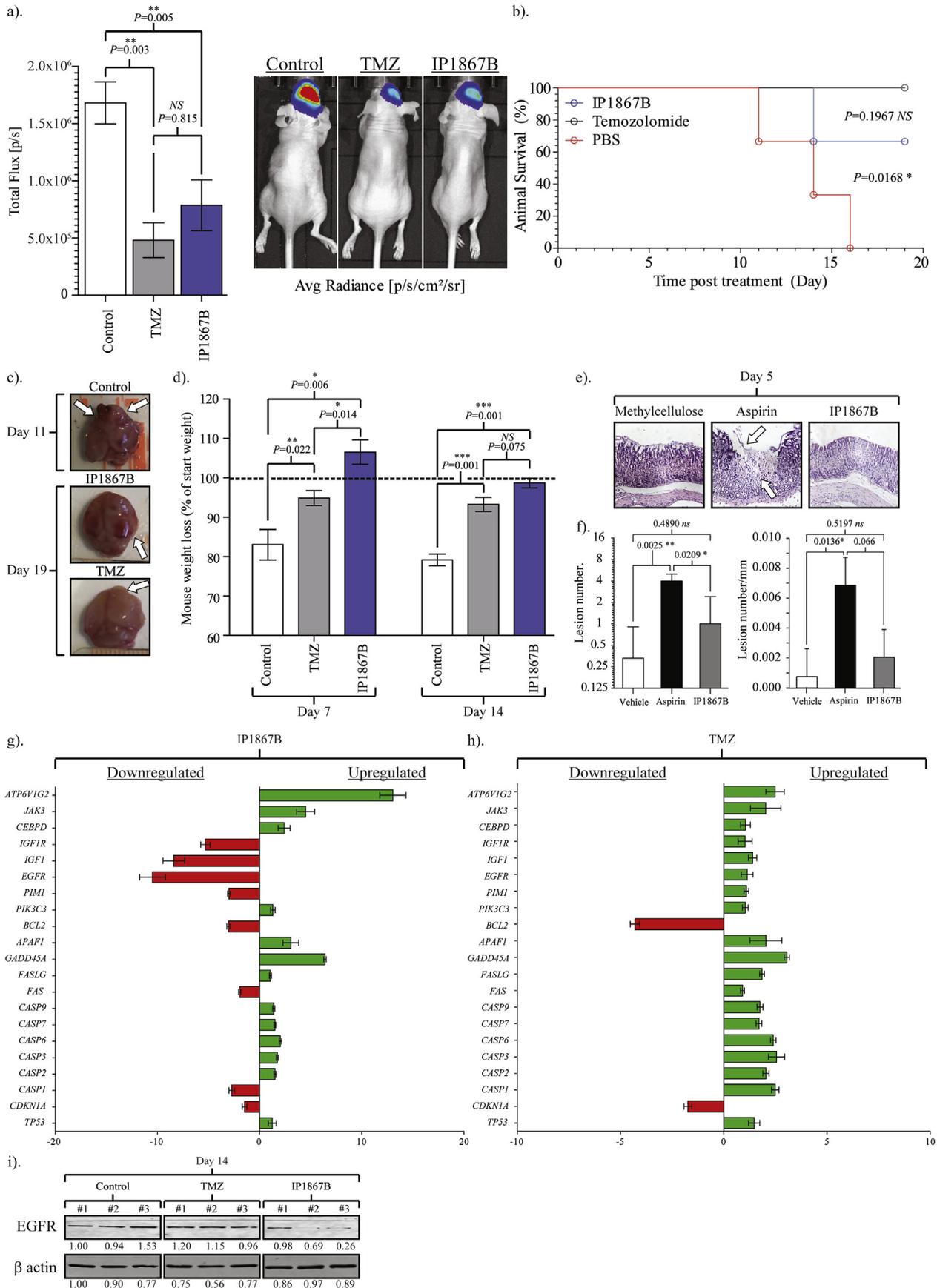


Fig. 3. IP1867B directs significant cell death and suppression of key inflammatory networks in aHGGs. a). Representative immunoblots for caspase 3 cleavage following IP1867B or TMZ exposure. b). Total p53 accumulation and activation in aHGG cells post-IP1867B treatment. c). FACS analysis 96 h post-drug treatment. d-f). Volcano plots for cell death pathway, IL6/STAT3 and NF- κ B signalling networks following IP1867B treatment of UP-029 aHGG cells. g). Key up and down-regulated gene expression changes identified in d-f 24 h post-IP1867B treatment of UP-029 aHGG cells. $n = 3$, error bars indicate \pm StDev with P values shown.



(caption on next page)

Fig. 5. IP1867B IP treatment induces significant reduction of intracranial tumours. a) Overall luciferase signal at day 19 following IP treatment of U87-MG tumour bearing mice. Brackets indicate two-tailed ANOVA with *P* values shown for each. Significant reduction of U87-luciferase tumours 19 days post IP1867B treatment. Representative tumour bearing mouse bioluminescence images post treatment with vehicle only (NT), TMZ or IP1867B. b) Overall survival for each group following mock, TMZ or IP1867B IP administration. c) Representative whole brain images following PBS, IP1867B or TMZ treatment. White arrows highlight tumour/distortion following tumour growth d). Average mice weight following vehicle only, TMZ or IP1867B treatment on day 7 or day 19 post tumour establishment. Brackets indicate two-tailed ANOVA analysis with *P* values shown for each comparison. e). Representative microscopic images of the gastric mucosa tract following control, aspirin or IP1867B oral delivery. f). (left) Quantitative analysis of gastrointestinal mucosal lesion formation following control, aspirin or IP1867B oral delivery. (right) lesion number/mm following the same treatment regimen shown in e-f. g-h). Gene expression analysis in U87-MG tumours treated with either IP1867B or TMZ at day 19 *n* = 3, error bars indicate \pm StDev. i). Representative immunoblot for EGFR following *in vivo* treatment with each therapeutic at day 19.

pSer473 AKT (Fig. 4G). Neither Gefitinib nor AZD3759 had a significant impact on pSer473 AKT level, although IP1867B treatment noticeably reduced the level of pSer473 AKT. Concomitantly, we noted elevated FOXO3a-dependent gene expression post-IP1867B treatment (Fig. 4H). These data indicated that there was significant suppression of the inflammation response and the IGF1R network, including AKT, which together increased the effectiveness of EGFR inhibitors.

3.4. IP1867B effectively repressed aHGG growth in intracranial implanted tumours

The *in vivo* effectiveness of IP1867B was addressed using a U87-luciferase model. U87-MG-luciferase aHGG cells were intracranially implanted into *NOD/SCID* mice (six mice per group) and 7 days post-inoculation, luciferase activity was measured. Tumour-bearing mice were treated on day 1, 3, 5, 8, 10 and 12 by intraperitoneal (IP) injection of vehicle, IP1867B (30 mg/kg) or TMZ (50 mg/kg) (Fig. 5A). There was a significant reduction in luciferase expression in IP1867B treated mice and a significant increase in survival (Fig. 5B). We collected the brain and liver of each animal. We noted significant intracranial tumour in vehicle treated mice, in particular, gross disruption of brain architecture and disruption of the midline. TMZ and IP1867B treated brains showed “normal” brain structure (Fig. 5C). We carefully monitored animal weight and behaviour and noted there was no significant weight loss in IP1867B treatment group (Fig. 5D). A concern regarding high dose aspirin treatment was potential damage and lesion development within the gastrointestinal tract. In an independent (non-tumour bearing mouse study) we examined if IP1867B oral administration induced any gastric mucosal lesions (Fig. 5E). There were significantly less total mucosal membrane lesions in addition to significantly reduced lesion frequency (per mm) in IP1867B treated mice versus aspirin treated mice (Fig. 5F).

IP1867B treatment of tumour bearing mice significantly reduced *IGF1* and *IGF1R* *in vivo* expression and, in agreement with our *in vitro* data, there was a significant down regulation of multiple inflammatory genes in the treated tumours (Fig. 5G and H). There was an upregulation of *caspase 3* and *caspase 9* transcription in the IP1867B treated mice brains although this induction was not seen in the matched livers (data not shown). A number of apoptotic markers were upregulated in the brains and livers of TMZ treated mice. This was both tumour specific (as measured using human-specific primers) and systemic (determined using mouse specific primers). Following IP1867B exposure, there was a significant downregulation of EGFR protein expression in the tumours of treated mice (Fig. 5I). This downregulation was not observed following mock or TMZ treatment regimens.

4. Discussion

The gold standard treatment for aHGG patients is the “Stupp protocol” with temozolomide. Despite this aggressive multi-modal regimen, patient prognosis remains poor with median survival of approximately 15 months.

The nonsteroidal anti-inflammatory agent aspirin is widely used for preventing and treating cardiovascular and cerebrovascular diseases while recent cohort analysis has suggested that aspirin may prevent a range of cancers (including colon, gastric and pancreatic cancer)

[30–33]. There is accumulating evidence that aspirin may act in different cell types, including epithelial cells, tumour cells, endothelial cells, platelet, and immune cells. Consequently, aspirin could act on multiple cancer hallmarks including cell growth, metastasis, angiogenesis and inflammation. There are noted side effects during long-term low dose aspirin treatment regimens that include nausea, vomiting, and abdominal pain. An important clinical complication following long-term aspirin administration is gastrointestinal injury, in particular gastroduodenal destruction, ulceration, and haemorrhage(s) [34]. The risk of major bleeding following aspirin treatment is higher in patients aged 75 years or older [34]. The median age at diagnosis of aHGG is 64 [35,36] where the incidence increases with age peaking between 75 and 84 years and this could present a major clinical obstacle for standard aspirin to be considered as a preventative or combinational therapy for aHGG patients.

Here we evaluated, IP1867B, a “true liquid” aspirin formulation. IP1867B allowed a higher concentration of ASA to be delivered *in vitro*, directed a potent anti-aHGG response and was well tolerated by non-neoplastic astrocytes. Compared to each excipient component, IP1867B was synergistic and directed a potent anti-aHGG response independent of *MGMT* promoter methylation. There was induction of apoptosis following IP1867B treatment, consistent with caspase 3 cleavage. One of our biopsy-derived aHGG cells had no detectable p53 protein although was sensitive to IP1867B, suggesting that the mechanism of action was likely p53-independent. There was significant suppression of the NF- κ B and IL6/STAT3 pathways and in particular suppression of *EGFR* transcription post-IP1867B exposure. We validated the suppression of *EGFR* transcription and noted the concomitant reduction of EGFR protein expression. A number of anti-EGFR clinical trials have been instigated and for aHGG [37,38]. Our data suggested that IP1867B could complement EGFR inhibitors. We evaluated Gefitinib and AZD3759 alone and in combination with IP1867B. Dual treatment with IP1867B significantly increased the potency of two EGFR inhibitors and significantly reduced ser473-AKT phosphorylation in two *in vitro* aHGG models. We examined the transcription of *IGF1* and *IGF1R* as these have been implicated in EGFR signalling and could inhibit AKT activation. IP1867B treatment significantly reduced *IGF1* and *IGF1R* transcription. These data were highly unexpected and compelling as to date there are no blood-brain barrier permissive anti-IGF1 and IGF1R treatments. Metabolic complications such as obesity, hyperglycemia, and type 2 diabetes are associated with poor outcomes in aHGG patients. To control peritumoral edema, high-dose steroids usage is common which can result in *de novo* diabetic symptoms. These could activate IGF1 and IGF1R in aHGG cells. The administration of IP1867B could significantly attenuate these treatment complications noted in aHGG patients.

IP1867B showed discernibly less propensity in producing gastric injury and induced significantly less gastric mucosal lesions compared to conventional aspirin. There was no significant difference between vehicle and IP1867B treated mice. The IP administration of IP1867B caused a significant reduction in overall intracranial tumour mass and was well tolerated. Tumour-bearing mice showed no significant weight loss or behavioural changes indicative of tumour burden. Both *in vitro* and *in vivo* IP1867B treatment revealed a reduction of EGFR in addition to reduced *IGF1* and *IGF1R* expression.

These data warrant follow-up in combination with EGFR inhibitors and validation of IP1867B as a putative IGF1 and IGF1R inhibitor. Care

is required as IGF1 and IGF1R have important functions in metabolism thus the prolonged blockade of this pathway may be associated with adverse effects. Agents that can penetrate the blood-brain barrier and complement conventional and novel therapeutics are of significant interest and warrant follow-up investigation.

Author contribution

KM, CC, MR, PAM, PM, SN, DMP, CAD, AH and RH conducted data curation and formal data analysis. GJP and RH devised the study. RH wrote the original draft. All authors revised/edited manuscript. Project administration was conducted by RH. Funding was awarded to GJP and RH.

Conflicts of interest

All authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2019.05.028>.

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