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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 aggress[illegible] primary brain [illegible]mours with local invasive growth and poor clinical prognosis. Clinical outcome is compounded [illegible]sistan[illegible] standard and novel therapeutics. We have evaluated reformulated aspirin (IP[illegible]) alone and in combination with conventional and novel anti-aHGG agents. We show that recent biopsy-[illegible]ived [illegible]odels were highly resistant to conventional therapeutics although show sensitivity to IP1867B, a r[illegible]rmulated [illegible]pirin. IP1867B treatment mediated a potent suppression of the IL6/STAT3 and NF-κB path[illegible]ys a[illegible] observed a significant reduction in EGFR transcription and protein expression. We observed the l[illegible] the insulin-like growth factor 1 and insulin-like growth factor 1 receptor expression[illegible]e transcri[illegible]nd protein level post IP1867B treatment. This increased sensitivity to EGFR inhibito[illegible]n vivo, [illegible]867B was [illegible] well tolerated, had little-to-no gastric lesions versus aspirin and, directed a signifi[illegible]t reductio[illegible]f tumour b[illegible]en with suppression of EGFR, IGF1 and IGFR1. With EGFR inhibitors, we not[illegible] potent sy[illegible]ase in aHGG cells. These data provide a rationale for further investigation of IP18[illegible] with a[illegible]mber of anti-EGFR agents currently being evaluated in the clinic.

1. Introduction

In 2017, 80,000 patients in [illegible] US and 11,400 [illegible]he UK were diagnosed with a primary bra[illegible] tumour [1,2]. Paediat[illegible] high grade glioma frequency in the U[illegible]are 5.7 c[illegible]ses per 100,000. Adult HGG treatment comprises su[illegible]cal resec[illegible]n and combined radio- and chemo-therapy [3,4]. Th[illegible]ndard [illegible]hemothera[illegible]utic is temozolomide (Temodal < SUP > TM < /[illegible] (TMZ), [illegible] oral DNA alkylating agent [5–7]. TM[illegible] radioth[illegible]y (the [illegible]upp protocol") demonstrated signific[illegible]t pati[illegible] survival [illegible] versus radiotherapy alone, showing a m[illegible]an incr[illegible]e in survival of 2.5 months [3,4,8]. Cohort analysis r[illegible]ealed a di[illegible]rible clinical response only in adult high grade glio[illegible]aHGG [illegible]patients exhibiting promoter methylation of *O6-methylguanine* [illegible] *methyltransferase* (*MGMT*) [9]. Irrespective of *MGMT* promoter [illegible]thylation, almost all aHGG patients exhibited eventual disease prog[illegible]ion. 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-κB, 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[illegible]vinylpyrrolidine (V0400000) and aspirin (Sigma-Aldrich). Gemcitabine (S1[illegible]4), Gefitinib or AZD3759 (SelleckChem). Adult glioma cell line U[illegible]MG was obtained from the ATCC and maintained in D[illegible]M supplemented with 10% heat inactivated FCS (Sigma-Aldrich). Novel [illegible]HG biopsy-lines UP-029, SEBTA-003, SEBTA-023 and SE[illegible]A-025 were cultured in DMEM medium. CC2565 non-neoplastic astrocytes (Lonza) were maintained in astrocyte growth [illegible] supplemented with SingleQuots™ (CC-3187 Lonza) including (CC-[illegible]3): rhEGF, insulin, ascorbic acid, L-glutamine. Cells were cultured under normoxic (21%) or hypoxic (1%) O_2 conditions.

2.3. MTS cell viability assay

5×10^3 cells were seeded [illegible]plicate in a [illegible] well plate. 24 h post seeding, cell lines [illegible]ated with each agent. CellTiter 96® AQueous One Solution M[illegible]S (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)) Reagent (G3580 Promega) was added and [illegible]t 490 nm recorded.

2.4. Western blot analysis

Cells were harvested and lysed using RIPA buffer (89900, ThermoFisher) 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 Li[illegible]06 (Roche) (iTaq SYBR Green, 1725120 BioRad). Primer sequences [illegible]wn in Supplemental Table 1. Data analysis was ca[illegible]ed out using the $^{-\Delta\Delta CT}$ method [24].

2.6. RT² pathway transcriptomics and analysis

Total RNA was extracted per [illegible]ndition (N[illegible]6hr IP186[illegible] using the RNeasy Plus Mini Kit (Qiagen, [illegible]including [illegible]nomic DNA removal. The quantified by RNA 6000 Nano Ki[illegible]rile[illegible]UK). 0.5 μg RNA was used for RT² cDNA (Qiagen, U[illegible]cDNA w[illegible]ded on th[illegible]ell death Pathway Finder (PAHS-212Z), N[illegible]κB signalling [illegible]y (Qi[illegible]n, PAHS-025Z) and IL6/STAT3 signalli[illegible]hway (PAHS-160[illegible]ates were run using a Light Cycler 96 [illegible]che, [illegible]. The CT values were obtained and fold changes determined by $2^{-\Delta\Delta}$[illegible]4].

2.7. In vivo tumour efficacy

25[illegible]000 U87-lucifer[illegible] cells were inoculated (intra-cranially) into *NOD/SCID* mice (aged 10[illegible]2 weeks). Animals were grouped based on lucifer[illegible] imaging. Treat[illegible]ent was initiated every three days via IP injection[illegible]BS, TMZ 50[illegible]g/kg/day or IP1867B 30 mg/kg/day. *In vivo* bioluminesc[illegible]g was performed once per week and all animals [illegible]e weighed with behaviour and neurological signs (including altered gait, [illegible] seizures and/or lethargy) monitored daily. This animal study was co[illegible]ducted 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, ($^*P \leq 0.05$), 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 EC_{50} 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

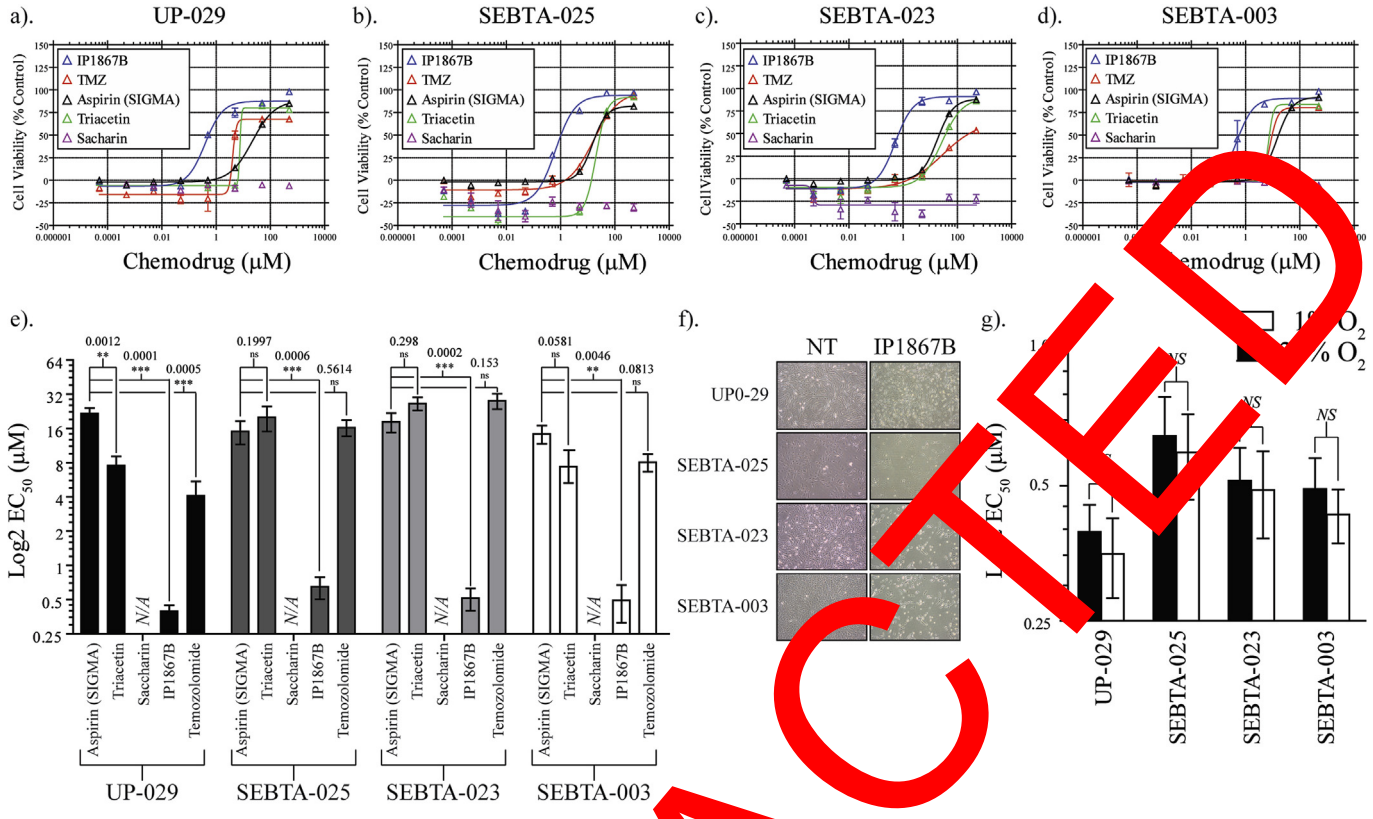

Fig. 1. IP1867B treatment of biopsy-derived aHGG cells significantly redu[ces] cel[l] [via]bility. a-d). Indicated aHGG cells (10,000 per well) were treated with varying dosages of each indicated compound and 96 h post treatment, MTS (Promega) assays [were] conducted. $n = 3$, error bars indicate ± StDev. 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. $n = 3$ ± StDev. f). Representative microscopy images for each biopsy-derived aHGG cell line 24 h post IP1867B treatment (40× 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. $n = 3$ ± StDev.

maximal solubility of aspirin (SIGMA Aldrich) was 3 [mM] (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 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 alone 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. This was not observed post-treatment with any of the excipient components with only limited cell detachment/death post-TMZ treatment (Fig.

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 ≈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

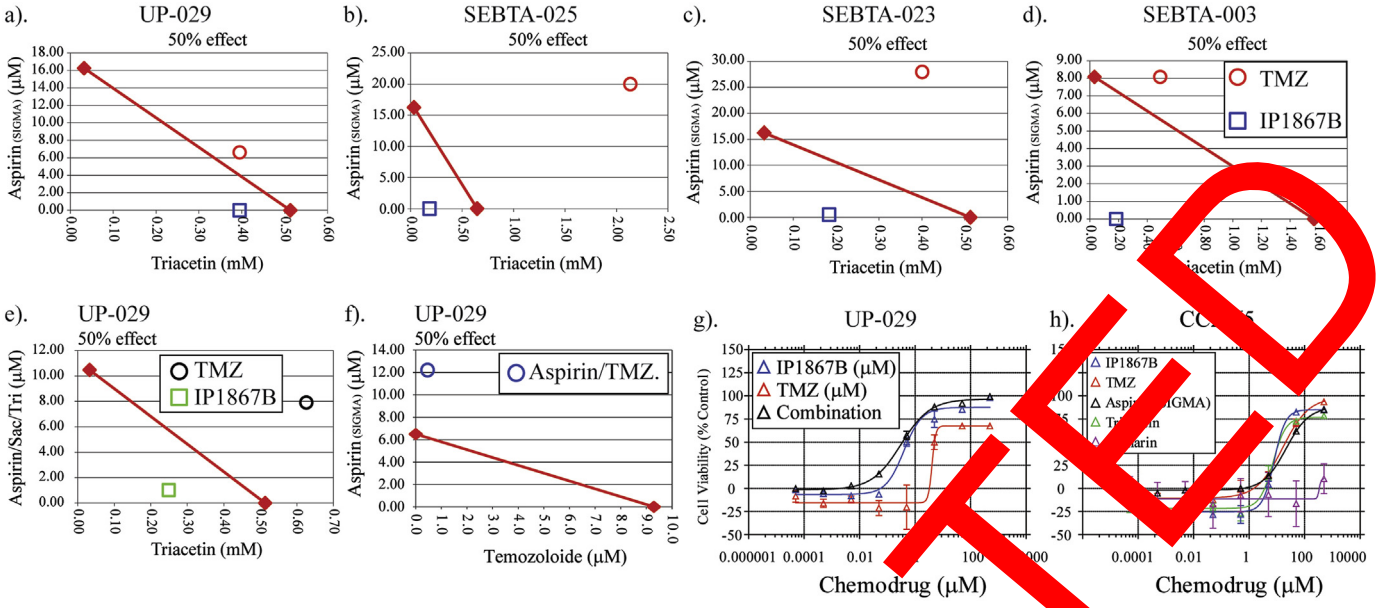

Fig. 2. IP1867B demonstrates synergy compared to each excipient component when pati ed aHGG cells are eated. a-d). Representative isobolograms from combination or single treatment of aspirin (SIGMA), triacetin, IP1867B or temo omide in aHGG cell lines. a). UP-029, b). SEBTA-025, c). SEBTA-003, d). SEBTA-023 cells were treated with each effective 50% concentration. IP1867B (or tem olomide) was adde 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 a illustrates the theo ical values resulting in additive effects. Data points below the line represent synergistic (CI ≤ 0.8) and above the line antagonistic (CI ≥ 1 interactions. n = e-f). Representative isobolograms from combination or single treatment of aHGG cells with IP167B and TMZ or aspirin and TMZ. n = 3 g). R sentative isbolo am dose curves for UP-029 aHGG cells treated with each indicated agent. h). CC2565 cells (10,000 per well) were treated with varying dosag f each in ted compound and 96 h post treatment, MTS (Promega) assays were conducted. n = 3, error bars indicate ± 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 redu in overall viability. IP1867B showed cancer-selectivity (EC_{50} ncer tion(s) for aHGG was between 0.39 and 0.6458 μM an e CC25 EC_{50} was 10.16 μM) (P = 0.0038).

3.3. IP1867B treatment induces the potent suppressi of NF-κB IGF1R

We examined how IP1867B could media s response. We te if there was significant caspase 3 cleavage llow reatment with the IP1867B (Fig. 3A). There was the signi ant detecti f cleaved caspase 3 at 96 h post-treatment. We q stioned if there p53 accumulation and activation. We did see discernible p53 ac mulation although noted p53 phosphory ion (Ser15 and Ser46) (Fig. 3B). In SEBTA-023 aHGG cells we w unable t etect any p53. To complement this study, each aHGG line w exposed t IP1867B or TMZ (100 μM) and at 24 and 96 h p tment FA analysis was conducted (Fig. 3C). We d ed signi t accu ation of asub-G_1 cell population consiste with death p IP67B treatment. At 96 h post-treatment, Z exposu also increa the sub-G_1 cell population, however s was signi antly lower compared to IP1867B treated aHGG cells.

We evaluated GG transcription response post IP1867B exposure and examined verse range of cell death networks, the NF-κB pathway, and the inflam ion response (Fig. 3D, E and F). Three housekeeping genes *Beta-2 icroglobulin* (*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 *interlukin (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 allmarl via downstream activation of STAT3. IL11 has been w o direct a more prominent role compared to IL6 in inflammatio associated cancers, suggesting a potential compensatory mecha m directed by aHGG following IP1867B exposure [25]. These data e the hypothesis that IP1867B could significantly complement a numb er of IL11 inhibitors (such as Bazedoxifene and mIL-11 Mutein) c ntly 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 potently 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.

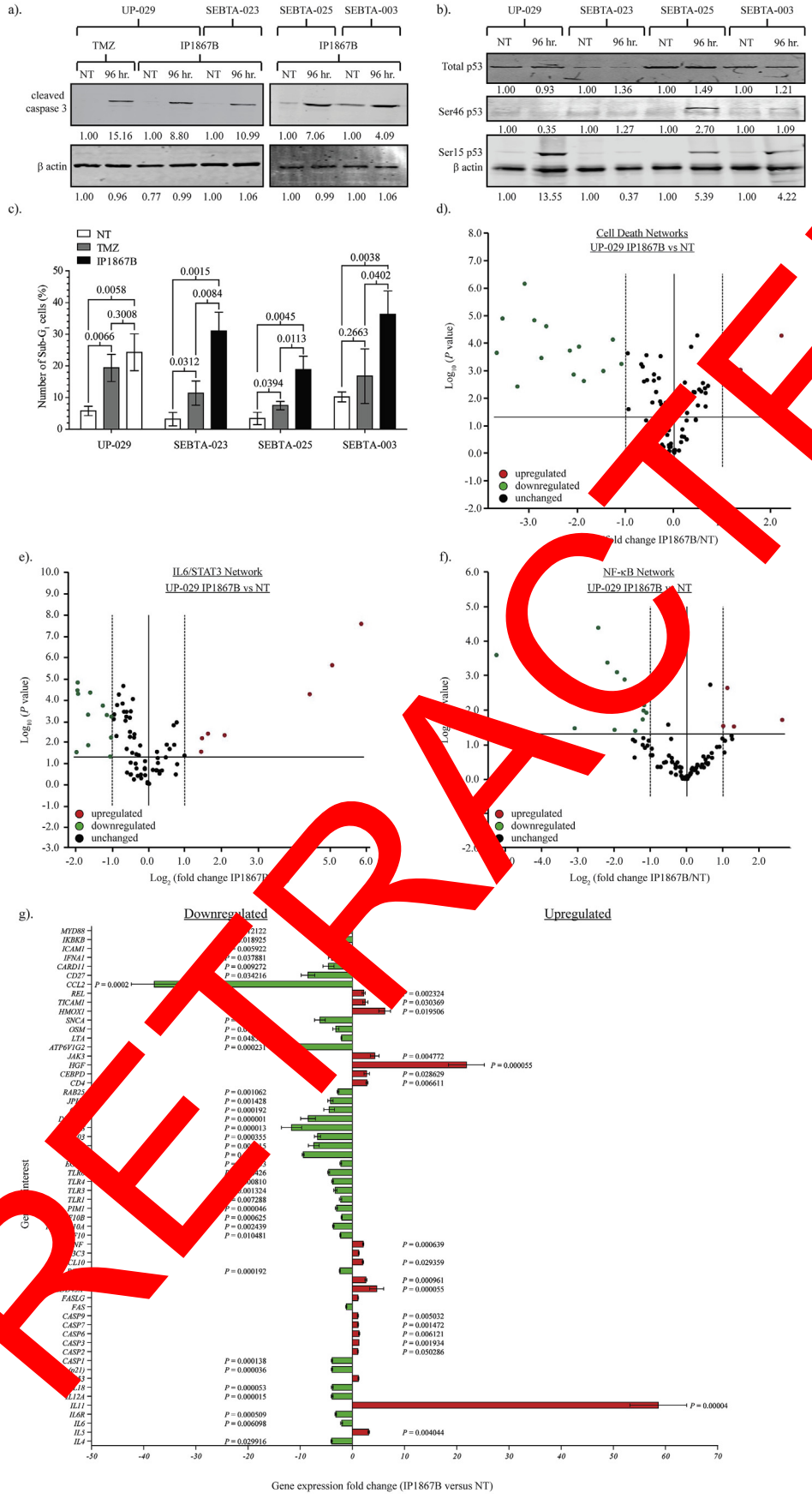

Fig. 3. IP1867B directs significant cell death and suppression of key inflammatory networks in aHGGs. a). Representative immunoblots for caspase 3 cleavage following IP1867B or TMZ exposure. b). Total p53 accumulation and activation in aHGG cells post-IP1867B treatment. c). FACS analysis 96 h post-drug treatment. d-f). Volcano plots for cell death pathway, IL6/STAT3 and NF-κB 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 ± StDev with P values shown.

Fig. 4. IP1867B treatme…gn…ntly sup…ses …FR, IGF1 and IGF1R expression and enhances anti-EGFR therapeutics. a-c) Gene expression analysis in each indicated aHGG c…line 24 h…st IP1867B …ent $n = 3$, error bars indicate ± StDev. d). Representative immunoblots for each indicated protein 24 h post IP1867B treatment. …). UP-029 a…GG cells (10,000 per well) were treated with varying dosages of each indicated compound and EGFR-inhibitor and 96 h post treatment, MTS (P…ega) assa…d. $n = 3$, error bars indicate ± StDev. g). Representative immunoblot for pSer473-AKT following treatment with each single or comb…on t…peutic. h). Expression of key FOXO3a-dependent genes 24 h post-treatment with each indicated therapeutic. $n = 3$, error bars indicate ± StDev. Two… ANOVA analysis conducted for each bracket. * indicates P value ≤ 0.05.

We treated our aHGG cell line…ith Gefitinib (EC_{50} 5 μM/L), AZD3759 (EC_{50} 50 nM) alone or in combination with IP1867B (Fig. 4E and F). Adult HGG cells showed limited sensitivity to Gefitinib and AZD3759 however, when used in combination with IP1867B, there was a significant increase in sensitivity to each agent. Each inhibitor was effective as a significant loss of detectable pY1068-EGFR was noted following exposure with Gefitnib and AZD3759 (Fig. 4G). EGFRvIII has been shown to be a direct regulator of STAT3. It was compelling that IP1867B treatment supressed this response [29]. IP1867B treatment in UP-029 and SEBTA-023 aHGG cells triggered a reduction in this network although we still detected, albeit reduced, EGFR and pY1068-EGFR. In untreated, Gefitinib, or AZD3759, treated aHGG cells, there was robust detection of IGF1R. Post-IP1867B treatment there was a significant reduction of IGF1R protein expression. The IGF1R pathway is an important receptor tyrosine kinase (RTK) in aHGG tumours [23]. In aHGG cells with high IGF1R expression, we detected high levels of

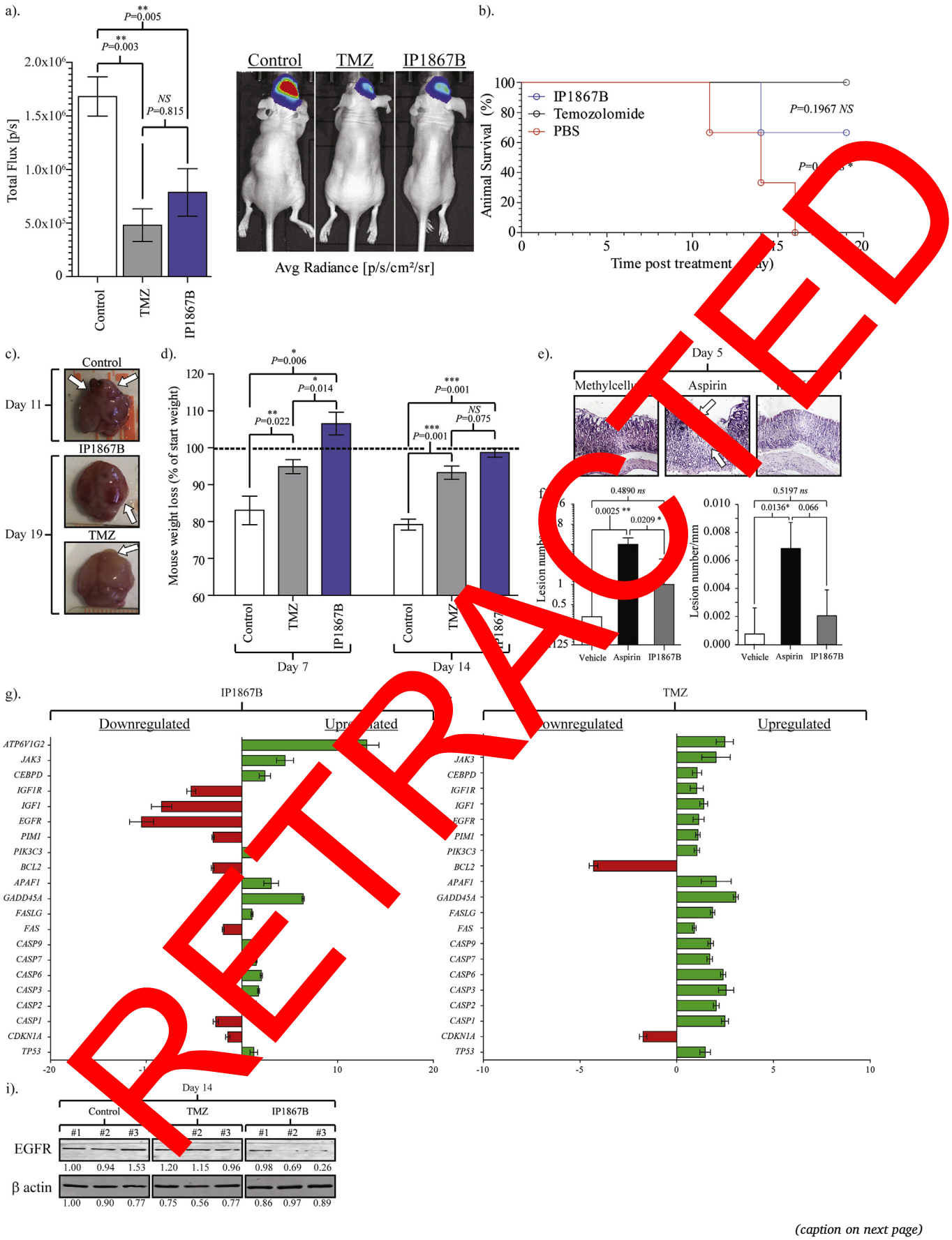

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Fig. 5. IP1867B IP treatment induces significant reduction of intracranial tumours. a) Overall luciferase signal at day 19 following IP treatment of U87-MG tumour bearing mice. Brackets indicate two-tailed ANOVA with *P* values shown for each. Significant reduction of U87-luciferase tumours 19 days post IP1867B treatment. Representative tumour bearing mouse bioilluminescence 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 ± 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 significantly reduced lesion frequency (per mm) in IP1867B treated 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 up-regulation of *caspase 3* and *caspase 9* transcript in the IP1867B treated 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. I). This down regulation was not observed following mock or TMZ treatment.

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 hemorrhage([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-κB 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 Getfinib 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 1P1867B 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.

References

[1] D.N. Louis, A. Perry, G. Reifenberger, A. von Deimling, D. Figarella-Br… W.K. Cavenee, H. Ohgaki, O.D. Wiestler, P. Kleihues, D.W. Ellison, The 20… health organization classification of tumors of the central nerv… system: a s… mary, Acta Neuropathol. 131 (2016) 803–820, https://doi.o…0.1007/s00401-016-1545-1.

[2] A. Pace, L. Dirven, J.A.F. Koekkoek, H. Golla, J. Fleming, R… C. Marosi, E. Le Rhun, R. Grant, K. Oliver, I. Oberg, H.J. Bulbeck, A.G… oney, P… rikkson, H.R.W. Pasman, S. Oberndorfer, M. Weller, M.J.B. Tap…oorn, Europe… sociation of Neuro-Oncology palliative care task force, Eur…ean Association for …e-Oncology (EANO) guidelines for palliative care … dults with glioma, Lancet … ol. 18 (2017) e330–e340, https://doi.org/10.10…S1470-2045(17)30345-5.

[3] R. Stupp, M.E. Hegi, W.P. Mason, M.J. va…n Bent, M. …Taphoorn, R.C. Janzer, S.K. Ludwin, A. Allgeier, B. Fisher, K. Be…ger, P. Hau, … Brandes, J. Gijtenbeek, C. Marosi, C.J. Vecht, K. Mokhtari, P. …seling, S. …, E. Eisenhauer, T. Gorlia, M. Weller, D. Lacombe, J.G. Cairncross, … Miri…hoff, Europea…rganisation for Research and treatment of cancer brain … and radiation …ology groups, National cancer institute of Can…linical trial…up, effects …diotherapy with concomitant and adjuvant t… versus ra…heran…one on survival in glioblastoma in a random… phase I… udy: 5-year … of the EORTC-NCIC trial, Lancet Oncol. 10 …09) 459–46… https://doi.org/…1016/S1470-2045(09)70025-7.

[4] R. Stupp, W.P. Mas… J.J. van de… B. Fisher, M.J.B. Taphoorn, K. Belanger, A.A. Bran… S. M…i, U. Bogdahn, J. Curschmann, R.C. Janzer, S.K. Ludwin, T. Gorlia, A. …, D. Lacombe, J.G. Cairncross, E. Eisenhauer, R.O. Mirimanoff, European or…tion for Research and treatment of cancer brain tumor and radiotherapy groups, …nal cancer institute of Canada clinical trials group, radiotherapy plus concomitan… adjuvant temozolomide for glioblastoma, N. Engl. J. Med. 352 (2005) 987–996, https://doi.org/10.1056/NEJMoa043330.

[5] A. Sankar, D.G. Thomas, J.L. Darling, Sensitivity of short-term cultures derived from human malignant glioma to the anti-cancer drug temozolomide, Anti Canccer Drugs 10 (1999) 179–185 http://www.ncbi.nlm.nih.gov/pubmed/10211548 , Accessed date: 5 December 2018.

[6] H.S. Friedman, S.P. Johnson, Q. Dong, S.C. Schold, B.K. Rasheed, S.H. Bigner, F. Ali-Osman, E. Dolan, O.M. Colvin, P. Houghton, G. Germain, J.T. Drummond, S. Keir, S. Marcelli, D.D. Bigner, P. Modrich, Methylator resistance mediated by mismatch repair deficiency in a glioblastoma multiforme xenograft, Cancer Res. 57 (1997) 2933–2936 http://www.ncbi.nlm.nih.gov/pubmed/9230204 , Accessed date: 5 December 2018.

[7] J. Plowman, W.R. Waud, A.D. Koutsoukos, L. V Rubinstein, T.D. Moore, M.R. Grever, Preclinical antitumor activity of temozolomide in mice: efficacy against human brain tumor xenografts and synergism with 1,3-bis(2-chloroethyl)-1-nitrosourea, Cancer Res. 54 (1994) 3793–3799 http://www.ncbi.nlm.nih.gov/pubmed/8033099 , Accessed date: 5 December 2018.

[8] R. Stupp, P.-Y. Dietrich, S. Ostermann Kraljevic, A. Pica, I. Maillard, P. Maeder, R. Meuli, R. Janzer, G. Pizzolato, R. Miralbell, F. Porchet, L. Regli, N. de Tribolet, R.O. Mirimanoff, S. Leyvraz, Promising survival for patients with newly di…nosed glioblastoma multiforme treated with concomitant radiation plus te… followed by adjuvant temozolomide, J. Clin. Oncol. 20 (2002) 137…382, https://doi.org/10.1200/JCO.2002.20.5.1375.

[9] M.E. Hegi, A.-C. Diserens, T. Gorlia, M.-F. Hamou, N. de Tri…t, M. Weller, J.M. Kros, J.A. Hainfellner, W. Mason, L. Mariani, J.E.C. Bro…y, P. Hau, R.O. Mirimanoff, J.G. Cairncross, R.C. Janzer, R. Stupp, *MGMT* g… ilencing and benefit from temozolomide in glioblastoma, N. Engl. J… d. 352 (20…997–1003, https://doi.org/10.1056/NEJMoa043331.

[10] S. Pushpakom, F. Iorio, P.A. Eyers, K.J. Escott, S… pper, A. Wells, A. Dol… T. Guilliams, J. Latimer, C. McNamee, A. Norr… Sanseau, … avalla, M. Pirmohamed, Drug repurposing: progress… allenges an… commendations, Nat. Rev. Drug Discov. (2018), https://doi.o…0.1038/…2018.168.

[11] C. Abbruzzese, S. Matteoni, M. Signore, A. Card…K…n, J.D. Glick…, M.G. Paggi, Drug repurposing for the …tment of g…stoma multif…e, J. Exp. Clin. Cancer Res. 36 (2017) 169, h…//doi.org/10.1…13046-…0642-x.

[12] C. Seliger, P. Hau, Drug repurpos…f metabolic agents in…r… glioma, Int. J. Mol. Sci. 19 (2018) 2768, htt…/10.3390/ijms1909…

[13] C.A. Dinarello, Anti-inflam…ry age…resent and future, Cell 140 (2010) 935–950, https://doi.org/1…1016/j.cell.2…02.043.

[14] F. Balkwill, K.A. Charles, A. Mantovani, Smo…g and polarized inflammation in the initiation and prom…on of malignant disea…ncer Cell 7 (2005) 211–217, https://doi.org/10…r.2005.02.013.

[15] M.D. Holmes, W… Chen, L. Li, E. Hertzmark, D. Spiegelman, S.E. Hankinson, Aspirin intake…d survival after breas… cancer, J. Clin. Oncol. 28 (2010) 1467–1472, h…s://doi.org/10.1200…O.2009.22.7918.

[16] E.K.S.M. Leer…s, H. Westdorp, R.J. …ggemann, J. Loeffen, C. Kratz, J. Burn, N. Hoogerbrug…M.C.J. Jongmans, C…er prevention by aspirin in children with constitutional m…tch repair deficie…y (CMMRD), Eur. J. Hum. Genet. 26 (2018) 1417–1423, https…org/10.10…41431-018-0197-0.

[17] A.T. Chan, S. Ogino, C… spirin use and survival after diagnosis of color-…er, J. Am. Med. Assoc. 302 (2009) 649, https://doi.org/10.1001/jama.…09.11…

[18] C…adeh, K.P… Aldape, EGFR and EGFRvIII in glioblastoma: partners in cr… Canc…ell 24 (2013) 403–404, https://doi.org/10.1016/j.ccr.2013.09.017.

[19] A.B…ip…ger, D. Suki, D. Yang, W. Shi, K. Aldape, The natural history of EGFR and E…III in glioblastoma patients, J. Transl. Med. 3 (2005) 38, https://doi.org/10.118…479-5876-3-38.

[20] R. Nishi…a, X.D. Ji, R.C. Harmon, C.S. Lazar, G.N. Gill, W.K. Cavenee, H.J. Huan… mutant epidermal growth factor receptor common in human glioma …fers enhanced tumorigenicity, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) …1 http://www.ncbi.nlm.nih.gov/pubmed/8052651 , Accessed date: 25 October 2018.

[21] E. Cerami, J. Gao, U. Dogrusoz, B.E. Gross, S.O. Sumer, B.A. Aksoy, A. Jacobsen, C.J. Byrne, M.L. Heuer, E. Larsson, Y. Antipin, B. Reva, A.P. Goldberg, C. Sander, N. Schultz, The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data, Cancer Discov. 2 (2012) 401–404, https://doi.org/10.1158/2159-8290.CD-12-0095.

[22] Y. Gong, Y. Ma, M. Sinyuk, S. Loganathan, R.C. Thompson, J.N. Sarkaria, W. Chen, J.D. Lathia, B.C. Mobley, S.W. Clark, J. Wang, Insulin-mediated signaling promotes proliferation and survival of glioblastoma through Akt activation, Neuro Oncol. 18 (2016) 48–57, https://doi.org/10.1093/neuonc/nov096.

[23] Y. Ma, N. Tang, R.C. Thompson, B.C. Mobley, S.W. Clark, J.N. Sarkaria, J. Wang, InsR/IGF1R pathway mediates resistance to EGFR inhibitors in glioblastoma, Clin. Cancer Res. 22 (2016) 1767–1776, https://doi.org/10.1158/1078-0432.CCR-15-1677.

[24] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[25] T.L. Putoczki, S. Thiem, A. Loving, R.A. Busuttil, N.J. Wilson, P.K. Ziegler, P.M. Nguyen, A. Preaudet, R. Farid, K.M. Edwards, Y. Boglev, R.B. Luwor, A. Jarnicki, D. Horst, A. Boussioutas, J.K. Heath, O.M. Sieber, I. Pleines, B.T. Kile, A. Nash, F.R. Greten, B.S. McKenzie, M. Ernst, Interleukin-11 is the dominant IL-6 family cytokine during gastrointestinal tumorigenesis and can Be targeted therapeutically, Cancer Cell 24 (2013) 257–271, https://doi.org/10.1016/j.ccr.2013.06.017.

[26] X. Wu, H. Xiao, C. Li, J. Lin, Abstract 186: FDA approved drug Bazedoxifene as a novel inhibitor of IL 6 and IL 11/GP130 signaling for osteosarcoma therapy, Cancer Res. 77 (2017), https://doi.org/10.1158/1538-7445.AM2017-186 186–186.

[27] S.W. Fanning, R. Jeselsohn, V. Dharmarajan, C.G. Mayne, M. Karimi, G. Buchwalter, R. Houtman, W. Toy, C.E. Fowler, R. Han, M. Lainé, K.E. Carlson, T.A. Martin, J. Nowak, J.C. Nwachukwu, D.J. Hosfield, S. Chandarlapaty, E. Tajkhorshid, K.W. Nettles, P.R. Griffin, Y. Shen, J.A. Katzenellenbogen, M. Brown, G.L. Greene, The SERM/SERD bazedoxifene disrupts ESR1 helix 12 to overcome acquired hormone resistance in breast cancer cells, Elife 7 (2018), https://doi.org/10.7554/eLife.37161.

[28] Y. Ma, N. Tang, R.C. Thompson, B.C. Mobley, S.W. Clark, J.N. Sarkaria, J. Wang, InsR/IGF1R pathway mediates resistance to EGFR inhibitors in glioblastoma, Clin. Cancer Res. 22 (2016) 1767–1776, https://doi.org/10.1158/1078-0432.CCR-15-1677.

[29] Q.-W. Fan, C.K. Cheng, W.C. Gustafson, E. Charron, P. Zipper, R.A. Wong, J. Chen, J. Lau, C. Knobbe-Thomsen, M. Weller, N. Jura, G. Reifenberger, K.M. Shokat, W.A. Weiss, EGFR phosphorylates tumor-derived EGFRvIII driving STAT3/5 and progression in glioblastoma, Cancer Cell 24 (2013) 438–449, https://doi.org/10.1016/j.ccr.2013.09.004.

[30] C. Bosetti, V. Rosato, S. Gallus, C. La Vecchia, Aspirin and urologic cancer risk: an update, Nat. Rev. Urol. 9 (2012) 102–110, https://doi.org/10.1038/nrurol.2011.219.

[31] M. Jiang, J. Dai, D. Gu, Q. Huang, L. Tian, Aspirin in pancreatic cancer: chemopreventive effects and therapeutic potentials, Biochim. Biophys. Acta Rev. Canc. 1866 (2016) 163–176, https://doi.org/10.1016/j.bbcan.2016.08.002.

[32] J. Cuzick, F. Otto, J.A. Baron, P.H. Brown, J. Burn, P. Greenwald, J. Jankowski, C. La Vecchia, F. Meyskens, H.J. Senn, M. Thun, Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement, Lancet Oncol. 10 (2009) 501–507, https://doi.org/10.1016/S1470-2045(09)70035-X.

[33] Y. Cao, R. Nishihara, K. Wu, M. Wang, S. Ogino, W.C. Willett, D. Spiegelman, C.S. Fuchs, E.L. Giovannucci, A.T. Chan, Population-wide impact of long-term use of aspirin and the risk for cancer, JAMA Oncol 2 (2016) 762–769, https://doi.org/10.1001/jamaoncol.2015.6.

[34] C.J. Lavie, C.W. Howd[illegible]. Scheiman, J. Tu[illegible]pper [illegible]trointestinal toxicity associated with long[illegible]n aspirin therapy: conse[illegible] and prevention, Curr. Probl. Cardiol. 42 [illegible]17) 146–16[illegible]ttps://doi.org/[illegible].1016/j.cpcardiol.2017.01.006.

[35] I. Chakrabarti, M.[illegible]kburn[illegible]Cozen, Y.-P.[illegible]ng, S. Preston-Martin, A population-based [illegible]cription[illegible]lastoma mul[illegible]me in Los Angeles County, 1974-1999, Ca[illegible] 104 (2005)[illegible]8–2806, ht[illegible]/doi.org/10.1002/cncr.21539.

[36] Q.T. O[illegible]m, H. Gittleman,[illegible]rah, A[illegible]dracek, Y. Chen, Y. Wolinsky, N.E[illegible]p, C. Kruchko, J.S. B[illegible]Sloan, CBTRUS statistical report: primary b[illegible]an[illegible]tral nervous system [illegible]ors diagnosed in the United States in 2006-[illegible]0, Neur[illegible]ol. 15 (Suppl 2) (2013) ii1–56, https://doi.org/10.1093/neuonc/not151.

[37] M. Patel, M.A. Vo[illegible]um, G.H. Barnett, R. Jalali, M.S. Ahluwalia, Molecular targeted therapy in recu[illegible]glioblastoma: current challenges and future directions, Expert Opin. Investig. Drugs 21 (2012) 1247–1266, https://doi.org/10.1517/13543784.2012.703177.

[38] T.E. Tayl[illegible]F.B. Furnari, W.K. Cavenee, Targeting EGFR for treatment of glioblastoma:[illegible]lecular basis to overcome resistance, Curr. Cancer Drug Targets 12 (2012) 197[illegible]09 http://www.ncbi.nlm.nih.gov/pubmed/22268382 , Accessed date: 27 Novem[illegible] 2018.