Interrogation of gossypol therapy in glioblastoma implementing cell line and patient-derived tumour models

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Background: Glioblastoma (GBM), being a highly vascularised and locally invasive tumour, is an attractive target for anti-angiogenic and anti-invasive therapies. The GBM/endothelial cell response to gossypol/temozolomide (TMZ) treatment was investigated with a particular aim to assess treatment effects on cancer hallmarks.

Methods: Cell viability, endothelial tube formation and GBM tumour cell invasion were variously assessed following combined treatment in vitro. The U87MG-luc2 subcutaneous xenograft model was used to investigate therapeutic response in vivo. Viable tumour response to treatment was interrogated using immunohistochemistry. Combined treatment protocols were also tested in primary GBM patient-derived cultures.

Results: An endothelial/GBM cell viability inhibitory effect, as well as an anti-angiogenic and anti-invasive response, to combined treatment have been demonstrated in vitro. A significantly greater anti-proliferative (P = 0.020, P = 0.030), anti-angiogenic (P = 0.040, P < 0.0001) and pro-apoptotic (P = 0.0083, P = 0.0149) response was observed when combined treatment was compared with single gossypol/TMZ treatment response, respectively. GBM cell line and patient-specific response to gossypol/TMZ treatment was observed.

Conclusions: Our results indicate that response to a combined gossypol/TMZ treatment is related to inhibition of tumour-associated angiogenesis, invasion and proliferation and warrants further investigation as a novel targeted GBM treatment strategy.

Glioblastoma (GBM) represents the most common and aggressive form of malignant brain tumour and is classified as a grade IV glioma by the World Health Organization. It is characterised by the presence of multifocal necrosis, microvascular proliferation, local tumour cell invasion and inherent resistance to apoptosis (Louis et al., 2007). Despite current standard of care, which includes surgery, radiation and chemotherapy with the alkylating agent temozolomide (TMZ), survival rates for GBM remains low. Median survival for patients is just 14 months from the time of diagnosis (Stupp et al., 2005, 2009).

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Angiogenesis has a crucial role in GBM tumour growth, maintenance and local spread throughout the brain. The formation of abnormal vasculature and local tumour cell invasion often results in resistance to conventional treatment (Onishi et al, 2011). Therefore, as we have previously suggested (Jarzabek et al, 2013b), the development of novel therapeutics, which inhibit more than one ‘hallmark of cancer’ (e.g., angiogenesis, GBM cell invasion, proliferation and/or apoptosis) and which act synergistically with standard treatment regimens, remains a critical objective.

One promising option may be inhibition of pro-survival B-cell lymphoma (Bcl-2) proteins (Azmi and Mohammad, 2009). It has been demonstrated that Bcl-2 proteins enhance tumour progression (through inhibition of apoptosis) (Adams and Cory, 2007; Chipuk et al, 2010), neo-angiogenesis (through induction of the hypoxia-inducible factor-1 transcription factor, endothelial cell survival and VEGF) (Biroccio et al, 2000; Fernandez et al, 2001; Kumar et al, 2008), tumour invasion (through induction of matrix metalloproteinases (MMPs)) (Wick et al, 2000; Choi et al, 2005) and resistance to chemotherapeutic agents, such as TMZ (Krakstad and Chekyna, 2010). Moreover, upregulated Bcl-2 expression has been detected in microvascular endothelial cells resulting in increased tumour angiogenesis and tumour growth (Nor et al, 2001).

Gossypol, a small molecule, naturally occurring BH3 mimetic, is a cotonseed-derived polyphenolic compound that has been extensively tested in humans as a male contraceptive agent (Qian et al, 2005), where it was shown to preferentially inhibit ethanol in sterile D-PBS (Sigma-Aldrich, St. Louis, MO, USA). For in vitro experiments, reagents were dissolved in DMSO at 100 mM stock solution. For in vivo studies, gossypol was solubilised in a vehicle solution of 10% ethanol in sterile dH2O (Xu et al, 2005), whereas TMZ was dissolved in DMSO at 100 mM stock solution.

Materials and methods

Reagents. Gossypol and TMZ were purchased from Sigma-Aldrich, St. Louis, MO, USA. For in vitro experiments, reagents were dissolved in DMSO at 100 mM stock solution. For in vivo studies, gossypol was solubilised in a vehicle solution of 10% ethanol in sterile dH2O (Xu et al, 2005), whereas TMZ was formulated in a vehicle solution of 10% DMSO in sterile D-PBS (Pedretti et al, 2010) as previously described. Both agents were freshly prepared before each administration.

Cell cultures. U87MG-luc2 GBM cell line was purchased from Caliper Life Science (A PerkinElmer Company, Hopkinton, MA, USA) and cultured in Eagle’s Minimum Essential Medium (EMEM) (Gibco, Invitrogen, Carlsbad, CA, USA). U251, U373 and U343 GBM cell lines were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD, USA) and cultured in high-glucose (4500 mg l−1) Dulbecco’s Modified Eagle’s Medium (DMEM) (Lonza, Basel, Switzerland). EMEM and DMEM were supplemented with 10% heat-inactivated foetal bovine serum (FBS), 1% l-glutamine (2 mM) and 1% penicillin/streptomycin (50 units ml−1), all from Sigma-Aldrich. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and maintained in Endothelial Cell Basal Medium-2 (EBM-2, Lonza). Each 500 ml of EBM-2 was supplemented with EGM-2 SingleQuots Kit (Lonza) consisting the following growth supplements: human epidermal growth factor (hEGF), 0.5 ml; vascular endothelial growth factor (VEGF), 0.5 ml; R3-insulin-like growth factor-1 (R3-IGF-1), 0.5 ml; ascorbic acid, 0.5 ml; hydrocortisone, 0.2 ml; human fibroblast growth factor-beta (hFGF-β), 2.0 ml; heparin (0.5 ml); fetal bovine serum (FBS), 25.0 ml; gentamycin/ampicillin-B (GA), 0.5 ml. All cells were maintained at 3% CO2 in humidified air with 5% CO2.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole (MTT)-based cell viability assay. Cell viability was determined using the MTT reagent (Sigma-Aldrich). GBM cell lines or HUVECs were seeded into 96-well cell culture plates (2000 GBM cells 100 μl−1 well−1 or 5000 HUVECs 100 μl−1 well−1). Following incubation (24 h), 100 μl of medium with or without test agent(s) was added into appropriate wells and incubated for the desired periods. Following incubation, 20 μl of MTT in D-PBS (5 mg ml−1) was added to each well. After 4 h, medium was replaced with 100 μl of DMSO in order to dissolve MTT formazan product. Absorbance was read on a spectrophotometer at 560 nm. Drug concentrations causing 50% cell growth inhibition (IC50 values) were calculated for single drug treatment via a sigmoidal dose–response curve using GraphPad Prism version 5.00 for Windows (Graphpad, San Diego, CA, USA). The dose–effect analysis by Chou and Talalay (1984) was used to assess the synergistic, additive and/or antagonistic cytotoxic effects of the combined treatment of GBM cells and HUVECs was assessed using the CompuSyn for Drug Combinations and for General Dose-Effec Analysis software (CompuSyn, Inc., Paramus, NJ, USA). The combination index (CI) was used to express synergism (CI < 1), additive effect (CI = 1) or antagonism (CI > 1).

In vitro endothelial cell capillary-like tube formation assay. HUVECs were seeded into 96-well cell culture plate coated with matrigel (BD Biosciences, Franklin Lakes, NJ, USA) in the absence/presence of agent(s). Cells were incubated for up to 20 h at 37 °C in CO2 incubator. Tube formation was observed under a phase-contrast microscope (Nikon, Tokyo, Japan) and a charge-coupled device camera (SPOT RT SE 6; Diagnostic Instruments, Inc., Sterling Heights, MI, USA) at × 200 magnification. Four randomly chosen microscopic fields per well were photographed with a digital camera. The number of branch-points per field-of-view was counted, and the length of tubes was quantified using the ImageJ 1.44 software (NIH, Bethesda, MD, USA).

3-D invasion assay. GBM cell line spheroids were formed using hanging drop method previously described (Del Duca et al, 2004). Following incubation (48 h), cell aggregates were transferred to a 100-mm dish coated with 2% agar filled with 10 ml of growth medium. Following incubation (48 h), spheroids were implanted.
into collagen gel (one sphere per well and 500 μl of collagen solution per well in 24-well plate). Chilled PureCol Bovine Collagen Product 3 mg ml⁻¹ (Nutacon BV, Leimuiden, The Netherlands) was mixed with 10-fold concentrated Dulbecco’s minimal essential medium (Sigma-Aldrich) and cold 0.1 M sodium hydroxide (Sigma-Aldrich) at a ratio of 8:1:1. pH was neutralised by adding 1 M NaOH (Sigma-Aldrich). Following incubation in 37 °C (1 h), 500 μl of complete growth medium was added to each well and returned to incubator. Gossypol (either with or without TMZ) was re-suspended in growth medium and added to the collagen gel matrix 2 days after implantation of spheroids into collagen gel (when invasion had already started). Cell invasion out of the spheroid was measured before treatment commenced (day 0) and on days: 2, 6, 8, 13 and 15, using a graded objective, a phase-contrast microscope (Nikon). Images were taken with a charge-coupled device camera (SPOT RT SE 6; Diagnostic Instruments, Inc.) at day 15. Alternatively, a 3-D matrigel invasion assay was performed as previously described (Kwiatkowska et al, 2012) with minor modifications. Briefly, 1 × 10⁴ of U87MG-luc2 cells were re-suspended in 50 μl of BD Matrigel and added to the top well (BD Falcon Cell culture inserts for 24-well plates, 8.0-μm pores, Translucent PET Membrane (BD Biosciences, Franklin Lakes, NJ, USA)). Following incubation at 37 °C for 30 min, 200 μl of serum-free growth medium with or without drug(s) was added to the top well and 700 μl of serum containing growth medium with or without drug(s) to the bottom well. After 48 h, cells attached to the bottom of insert were fixed and stained with 0.5% methylene blue dissolved in DHO (Sigma-Aldrich). Cells on the upper surface of the filter were wiped off with a Q-tip. Images were taken using a colour device camera.

GBM xenograft model and drug treatment. All animal experiments were licensed by the Department of Health and Children in Ireland. Study protocols were reviewed by the Animal Research Ethics Committee (AREC) at the University College Dublin. Female BALB C nu/nu mice (4–6 weeks) were procured at the SPF-grade Conway Institute Biotechnical Services (CIBS) Xenograft Facility from Harlan Laboratories, UK, Ltd, Bicester, UK. Mice were anesthetised with O₂/isoflurane mixture, and volumes estimated by the formula: a² × b × 0.5, where a and b are the shorter and longer diameter of the tumours, respectively. When tumours reached approximately 200 mm³ (approximately 28 days following inoculation), mice were randomised into treatment groups. No difference in mean tumour volume was observed among the treatment groups at day 0 before treatment commencement. Group 1 (n = 8) received 30 mg kg⁻¹ of gossypol by oral gavage (p.o.), using a regimen of daily dosing for 5 consecutive days, followed by a 2-day rest, before resuming dosing. Group 2 (n = 6) was treated with 7.5 mg kg⁻¹ of TMZ by intraperitoneal injection (i.p.) daily for 9 consecutive days. Group 3 (n = 8) received both gossypol and TMZ treatments using the same regimens as above. On days when both drugs were administered, TMZ was administered at least 5 h after gossypol. The control cohort received an equivalent volume of vehicle for gossypol (10% ethanol in sterile dH₂O) and TMZ (10% DMSO in sterile D-PBS). All animals were monitored for adverse effects. When tumours exceeded 15 mm in any of the diameters, animals were euthanised.

Histological examination and immunohistochemistry. Subcutaneous tumours were excised, rinsed twice in D-PBS and fixed in 4% formaldehyde for 48 h. Tissues were embedded in paraffin and 5-μm thick sections were cut. Sections were deparaffinised with histoclear II and re-hydrated according to standard histological procedures. Routine haematoxylin and eosin (H&E) staining was performed to facilitate histological evaluation by a veterinary pathologist. For immunohistochemistry, epitope retrieval was performed by microwave boiling for 20 min in 10 mM citrate buffer at pH 6.0. One hour blocking in 5% serum/TBS-T was used before primary antibody incubation (rabbit anti-human Ki67 (Millipore, Billerica, MA, USA, dilution 1:100) for 1 h in room temperature or goat anti-PECAM-1/C2 (Santa Cruz Biotechno-

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Gossypol acts synergistically with TMZ to inhibit endothelial cells (HUVECs) and GBM (U87-MG-luc2 and U343) cell lines. To investigate the dose-dependent cytotoxic effect of gossypol on GBM and endothelial cells and to estimate and compare IC₅₀ concentrations, four GBM cell lines (U87MG-luc2, U251, U373, U343) (Figure 1A) and HUVECs (Figure 1B) were treated with different concentrations of gossypol for 72 h. Mean IC₅₀ values, for each GBM cell line and endothelial cells, were obtained. Enhanced gossypol cytotoxicity was observed in HUVECs showing lowest IC₅₀ concentration (1.699 ± 0.178) when compared with IC₅₀ concentrations derived for four different GBM cell lines. Among the GBM cell lines tested, U251 and U373 exhibited higher IC₅₀ values following 72 h treatment (11.600 ± 4.353 and 10.120 ± 2.931, respectively) when compared with U343 and U87 cell lines (5.784 ± 0.458 and 5.689 ± 0.487, respectively) (Figure 1C).

To examine the sensitivity of GBM cells to combined gossypol/TMZ treatment, U87MG-luc2, U251, U373 and U343 cells were exposed to various concentrations of gossypol (3, 6 or 12 μM) either with or without differing concentrations of TMZ (100, 200 or 400 μM) for 96 h (Figure 2). Maximum growth inhibition (400 μM TMZ) did not exceed 52, 53, 27 or 52% in U87MG-luc2, U251, U343 or U373 cell lines, respectively. Addition of gossypol enhanced the anti-proliferative effect of TMZ, increasing growth inhibition to 89, 70, 63 or 82%, respectively, in U87MG-luc2, U251, U343 or U373 cell lines (one-way ANOVA, P < 0.05). Combining TMZ with gossypol (3 μM) potentiated the effect of gossypol alone (Figure 2A, C, E and G). Isobologram analysis was employed to further investigate whether observed gossypol and TMZ interactions were antagonistic, additive or synergistic. CI values <1 = or >1 indicate synergy, additive effect or antagonism, respectively. The greatest synergistic interaction was observed in the U343 cell line, some synergistic interaction was observed in U87MG-luc2 cell line, whereas U251 and U373 cell lines exhibited additive and antagonistic responses to gossypol/TMZ treatment (Figure 2B, D, F, H and K).

Next, sensitivity of HUVECs to combined gossypol/TMZ treatment was examined. HUVECs were exposed to various concentrations of gossypol (1.5, 3 or 6 μM) either with or without various concentrations of TMZ (50, 100 or 200 μM) for 72 h. As HUVECs are more sensitive to gossypol treatment, lower drug concentrations and shorter incubation times were used to assess treatment efficacy. Maximum growth inhibition with 200 μM of TMZ did not exceed 30%. Nevertheless, the addition of gossypol greatly enhanced the anti-proliferative effect of TMZ, causing up to 88% growth inhibition in HUVECs. Combining TMZ with 1.5 μM gossypol appeared to potentiate the effect of single-agent gossypol (Figure 2I). Similarly, in order to further analyse drug combination response in HUVECs, isobologram analysis was performed.

Analysis of CIs provided evidence that the majority of treatment conditions implemented in HUVEC studies promoted synergistic cell killing (Figure 2 and K).

Gossypol in combination with TMZ treatment inhibits HUVEC tube formation in vitro. In order to further examine the effects of gossypol/TMZ treatment on the final step of angiogenesis, (tubulogenesis), we employed an in vitro tubule-formation assay (Figure 3A–H). Tubule length (Figure 3I) and branch point (Figure 3J) analyses demonstrated significant inhibition of both tubule length and branch points in a dose-dependent manner following 20-h gossypol/TMZ treatment (P < 0.05 when compared with control). Moreover, comparison of combined gossypol/TMZ treatment with single-agent TMZ treatment revealed a significant decrease in the total tubule length (when gossypol was used at 3, 6
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Figure 2. Synergistic response to gossypol and TMZ combination therapy in GBM cell lines and primary endothelial cultures. (A–H) GBM and (I, J) endothelial cells were exposed to increasing concentrations of gossypol and/or TMZ for 96 or 72 h, respectively. Cell viability was assessed using the MTT assay. Each treatment condition was replicated in at least three wells and in three independent experiments. Means ± s.e.m. of three independent experiments are presented. *P < 0.05 compared with control (0 μM gossypol + 0 μM TMZ), one-way ANOVA and Tukey’s post-hoc test (A, C, E, G, I). The combination indices (CIs) corresponding to the gossypol/TMZ combinations tested in figures (A, C, E, G, I) were determined by the CompuSyn software (B, D, F, H, J, K). A normalised isobolograms (B, D, F, H, J) graphically represent the interaction between gossypol and TMZ, whereas (K) summary table lists all the CI values obtained from the analysis and their interpretations. The combination index (CI) was used to express synergism (CI < 1), additive effect (CI = 1) or antagonism (CI > 1).

Gossypol in combination with TMZ treatment inhibits GBM cell invasion in vitro. To investigate GBM cell invasion following gossypol ± TMZ treatment in vitro, we first employed a 3-D spheroid invasion assay using the U87MG-luc2 GBM cell line. In a control setting, U87MG-luc2 GBM cells demonstrated invasive activity reaching a distance of 3746.528 ± 127.625 μm from parent or 12 μM concentrations) and number of branch points (when gossypol was used at 6 or 12 μM concentrations). Comparison of combined gossypol/TMZ treatment with single gossypol treatment showed a significant decrease in the total tubule length and number of branch points when gossypol was implemented at concentrations of 3 and 6 μM (Figure 3I and J).
spheroid within 15 days. U87MG-luc2 cells treated with gossypol (3, 6 or 12 μM)/TMZ (100 μM) combination invaded to a lesser extent from the spheroid core through the collagen gel (Figure 4A–H). Significant anti-invasive effects of U87MG-luc2 cells treated with the combination regimen were observed in comparison to the invasion distance in control wells commencing from day 6 when 3 μM of gossypol was used and from day 4 when higher concentration (6 or 12 μM) of gossypol was used in combination with TMZ (100 μM) treatment. Moreover, spheroids treated with combined gossypol/TMZ treatment showed a significant difference when compared with single treatments as indicated in Figure 4I–K. Quantification of average invasion distance per day revealed that spheroids treated with the combination regimen gossypol (6 or 12 μM)/TMZ (100 μM) invaded at a significantly slower rate when compared with control (Figure 4L). The in vitro effect of gossypol treatment delivered in combination with TMZ on GBM cell invasion was further confirmed in 3-D matrigel invasion assay using trans-well Boyden chambers as shown in Supplementary Figure S1A–H.

Gossypol in combination with TMZ inhibits GBM cell proliferation, tumour angiogenesis and enhances apoptosis in an in vivo GBM setting. To investigate the in vivo effect of gossypol either with or without TMZ on tumour growth, proliferation, apoptosis and angiogenesis, we employed a subcutaneous GBM (U87MG-luc2) xenograft mouse model. Tumour growth was measured over time. Figure 5A presents study time points for drug combination treatment, BLI and caliper measurement. Figure 5B shows the mean tumour volume based on caliper measurements over time, and Figure 5C shows bioluminescence images of representative tumours at day 14. As revealed by caliper measurements, tumour volume in the vehicle-treated group of tumour architecture and a prominence of mononuclear giant cells were arranged in sheets and clusters in a fine fibrovascular stroma. Necrosis (most likely due to large tumour size/fast growing tumour) was often accompanied by a prominent influx of neutrophils, particularly at the junction of viable and necrotic tissue. In contrast, TMZ monotherapy and combined treatment showed marked distortion of tumour architecture and a prominence of mononuclear giant tumour cells with pleomorphic nuclei and an abundant eosinophilic cytoplasm. Moreover, the pattern of tight clustering of cells in a fibrovascular stroma (seen in vehicle and gossypol cohorts) was frequently lost in regions rich in mononuclear giant cells. Cells in these regions were more loosely arranged and lacking cluster architectures. We observed reduced necrosis in TMZ- and combination-treated tumours. Moreover, control tumours revealed numerous mitotic figure (> 5 mitotic figures per high power field (hpf)), whereas tumours treated with either single or combined therapies revealed decreased numbers of mitotic figures (0–3 mitotic figures per hpf) (Figure 5E (H&E images)). A mean count of Ki-67-positive nuclei of 24.86 ± 1.42 was

**Figure 3.** Gossypol in combination with TMZ inhibits endothelial cell tube formation in vitro. HUVECs were seeded on BD Matrigel in the presence or absence of gossypol (3, 6 or 12 μM) with or without TMZ (5 μM) and incubated for 20 h. Representative images of (A) control, (B) gossypol (3 μM)-, (C) gossypol (6 μM)-, (D) gossypol (12 μM)-, (E) TMZ (5 μM)-, (F) gossypol (3 μM)/TMZ (5 μM)-, (G) gossypol (6 μM)/TMZ (5 μM)- and (H) gossypol (12 μM)/TMZ (5 μM)-treated HUVECs were taken at 20 h time point. Scale bar = 100 μM (× 200 magnification). (I) Relative tubular length (percentage of control) and (J) relative number of branching points (percentage of control) were quantified. Each treatment condition was replicated in at least three wells and in three independent experiments. Mean ± s.e.m. are presented. *P < 0.05 compared with control, †P < 0.05 compared with TMZ treatment, ##P < 0.01 compared with gossypol treatment (one-way ANOVA and Tukey’s post-hoc test).
observed in vehicle-treated control animals. Single treatments, both gossypol and TMZ, exerted inhibitory effects on actively proliferating GBM cells, showing proliferation index of 15.01 ± 2.12 and 14.71 ± 1.66, respectively. Statistical analysis indicated that the decrease in proliferation in single gossypol and TMZ-treated animals was significant (P < 0.0001 compared with vehicle-treated tumours). Moreover, combined gossypol/TMZ treatment revealed a significantly greater decrease in proliferation compared with single treatments (9.071 ± 0.90, P < 0.0001 compared with vehicle-treated tumours, ##P < 0.0001 compared with TMZ treatment, ##P < 0.0001 compared with gossypol treatment, linear mixed model and ANOVA, post-hoc Tukey).

Figure 4. Gossypol in combination with TMZ inhibits GBM cell invasion in vitro. U87MG-luc2 spheroids implanted in collagen gel were exposed to gossypol ± TMZ treatment over 15 days. Representative images of (A) control, (B) TMZ (100 µM)-, (C) gossypol (3 µM)-, (D) gossypol (3 µM)/TMZ (100 µM)-, (E) gossypol (100 µM)-, (F) gossypol (6 µM)/TMZ (100 µM)-, (G) gossypol (12 µM)- and (H) gossypol (12 µM)/TMZ (100 µM)-treated spheroids were taken at day 15. Scale bar: 1000 µm (× 40 magnification). Invasion activity of U87MG-luc2 cells exposed to the absence or presence of (I) gossypol (3 µM)/TMZ (100 µM), (J) gossypol (6 µM)/TMZ (100 µM) and (K) gossypol (12 µM)/TMZ (100 µM) over 15 days of treatment, and (L) average invasion distance per day quantified as a percentage of control were quantified. Data presented are mean invasion distance ± s.e.m. of three independent experiments, *P < 0.05 compared with the control, #P < 0.05 compared with TMZ treatment, ##P < 0.05 compared with single treatments, both gossypol and TMZ, exerted inhibitory effects on actively proliferating GBM cells, showing proliferation index of 15.01 ± 2.12 and 14.71 ± 1.66, respectively. Statistical analysis indicated that the decrease in proliferation in single gossypol and TMZ-treated animals was significant (P < 0.0001 compared with vehicle-treated tumours). Moreover, combined gossypol/TMZ treatment revealed a significantly greater decrease in proliferation compared with single treatments (9.071 ± 0.90, P < 0.0001 compared with vehicle-treated tumours, ##P < 0.0001 compared with TMZ treatment, ##P < 0.0001 compared with gossypol treatment, linear mixed model and ANOVA, post-hoc Tukey).

Gossypol treatment enhances the efficacy of TMZ treatment in GBM patient-derived multicellular spheroids and GBM patient-derived neurosphere cultures. Recent studies have suggested that the response of GBM patient-derived cultures to anti-GBM treatment may better reflect potential therapeutic
Figure 5. Gossypol in combination with TMZ reduces tumour burden, inhibits angiogenesis and GBM cell proliferation and enhances apoptosis in vivo. (A) Drug combination treatment, BLI and Caliper measurements time points are presented. (B) Tumour volume and (C) tumour bioluminescence of each mouse was assessed at different time points. Mean tumour volumes ± s.e.m. (vehicle-treated (n = 6), gossypol 30 mg kg⁻¹-treated (n = 8), TMZ 7.5 mg kg⁻¹-treated (n = 6), combination gossypol/TMZ-treated (n = 8) groups) are shown over time (B). Representative bioluminescent images from second week of treatment are shown for each treatment group (C). (D) Incidence of animals (percentage of treatment cohorts) with or without necrotic lesions is shown in bar graph. (E) Representative images of H&E, anti-Ki67, TUNEL and anti-PECAM-1/CD31 stained xenograft sections treated with either vehicle, gossypol, TMZ or combination are shown. Scale bars = 50 μm (× 400 magnification, H&E), 100 μm (× 200 magnification, Ki67, TUNEL, PECAM-1/CD31). (F) Proliferation index (%), (G) TUNEL-positive cells (%) and (H) microvessel density (MVD) are shown. Error bars represent mean ± s.e.m. (vehicle-treated (n = 3), gossypol 30 mg kg⁻¹-treated (n = 3), TMZ 7.5 mg kg⁻¹-treated (n = 3), combination gossypol/TMZ-treated (n = 5) tumours); quantifications from five randomly chosen field of views per tumour (F–H). *P < 0.05 compared with vehicle, †P < 0.05 compared with TMZ 7.5 mg/kg, ‡P < 0.05 compared with gossypol 30 mg/kg (linear mixed model and ANOVA, post-hoc Tukey).
efficacy in vitro than adherent GBM cell lines (Singh et al, 2003, 2004; Johannessen et al, 2009). We therefore exposed GBM patient-derived cultures to treatment for 96 h. Viability following treatment was assessed using the MTT assay. In contrast to GBM cell lines (U87MG-luc2, U373, U343 and U251), GBM patient-derived multicellular spheroids (Figure 6A) and neurospheres (Figure 6B and C) exhibited greater resistance to TMZ treatment after 96 h. However, a significant reduction in cell viability was observed after single-agent gossypol treatment and combined gossypol/TMZ treatment when compared with control (Figure 6A–C).

**DISCUSSION**

We have explored the anti-angiogenic, pro-apoptotic, anti-proliferative and anti-invasive properties of treatment with the BH3 mimetic agent gossypol alone and in combination with TMZ, implementing in vitro and in vivo GBM/endothelial cell models.

In agreement with previous data (Coyle et al, 1994; Voss et al, 2010; Pang et al, 2011), we have shown a dose-dependent cytotoxic effect of gossypol on human GBM and endothelial cells (Figure 1). We tested gossypol alone in four GBM cell lines that differ in Mcl-1 and/or MGMT status (Supplementary Table S1). Our results revealed that U251 and U373 lines were less responsive to gossypol following 72 h treatment than other cell lines tested (U87MG-luc2 and U343). We believe that this may be partially due to Mcl-1 protein levels, which have previously been shown to be lower in U373 and U251 (Day et al, 2011). Inhibition of LDH may further contribute (Tuszynski and Cosus, 1984; Coyle et al, 1994; Seliger et al, 2013). Moreover, we have shown that endothelial cells exhibit greater sensitivity to single-agent gossypol treatment compared with GBM cells. Greater sensitivity of endothelial cells to gossypol was also observed previously when compared with sensitivity of human prostate cancer cells (Pang et al, 2011).

It has previously been reported that gossypol potentiates cell death induced by TMZ to a greater extent in cell lines with negative MGMT status (Voss et al, 2010). In order to further test this finding, we employed isobologram analysis to investigate the efficacy of combination therapy in four different GBM cell lines of known MGMT status (Figure 2). Greatest synergy between gossypol and TMZ was observed in the U343 line (MGMT-negative) as reported by Voss et al (2010). Some synergy was observed in the U87MG-luc2 cell line (MGMT-expressing) as also previously reported (Voss et al, 2010). Nevertheless, U373 (MGMT-low level) and U251 (MGMT-negative) GBM cell lines (Chahal et al, 2012) demonstrated additive and antagonistic response to combined drug treatment. Interestingly, it has previously been reported that U251 and U373 GBM cell line express low Mcl-1 levels (Day et al, 2011), whereas U87 and U343 cell lines exhibit high Mcl-1 expression levels (Hetschko et al, 2008; Day et al, 2011; Murphy et al, 2014). We hypothesise that Mcl-1 may impact sensitivity to gossypol regimens. The efficacy of combined treatment may also be dependent on LDH expression in a given cell line as in single-agent gossypol treatment (Le et al, 2010; Seliger et al, 2013). Combined gossypol and TMZ treatment in endothelial cells again exhibited greater sensitivity when compared with tumour cell lines. This effect was confirmed in an in vitro endothelial cell–tube-formation assay (Figure 3). Greater drug synergism in HUVECs was also observed. It has previously been reported that tumour-associated endothelial cells express higher levels of the Bcl-2 gene than normal endothelial cells (Kaneko et al, 2007), resulting in enhanced angiogenesis and tumour growth (Nor et al, 2001). Moreover, it has further been shown that gossypol modulates VEGF signalling-mediated angiogenesis partially contributing to in vivo suppression of prostate tumour growth. A potent inhibitory effect of gossypol on VEGF-induced endothelial cell motility was also observed in human prostate cancer xenografts (Pang et al, 2011).

We also investigated whether gossypol and TMZ combination could suppress the invasive behaviour of GBM cells. 3-D spheroid invasion and 3-D matrigel trans-well invasion assays were employed in order to mimic the complexity and pathophysiology of in vivo tumour invasion. A significant anti-invasion response to combined treatment was observed in both assays (Figure 4 and Supplementary Figure S1). Potent anti-invasive properties of combined gossypol/TMZ treatment observed in vitro may be due to perturbation of MMP-2 activity via inhibition of Bcl-2 (gossypol) and TGF-β2 (TMZ). This hypothesis is based on previously reported data, which indicates that (i) Bcl-2 proteins may promote tumour cell migration and invasion via induction of matrix metalloproteinase-2 (MMP-2) and cell surface urokinase-type plasminogen activator (uPA) (Wick et al, 1998; Choi et al, 2005; Trsicuoglo et al, 2005), (ii) transforming growth factor-beta 2
(TGF-β2) enables GBM cell infiltration via regulation of the expression of MMPs and αV/β3 integrin (Wick et al., 2002; Bruna et al., 2007) and that (iii) TMZ may inhibit (TGF-β2)-induced invasiveness of GSCs (Zhang et al., 2011). The molecular mechanism by which Bcl-2 promotes MMP-2 and uPA induction has not yet been elucidated. However, as Bcl-2 and other anti-apoptotic Bcl-2 family proteins are increasingly recognised as physiological regulators of mitochondrial fusion/fission dynamics, mitochondrial bioenergetics and ER/mitochondrial Ca2+ homeostasis (Kilbride and Prehn, 2013), these may be complex. Clearly, these ‘non-apoptotic’ functions of Bcl-2 and their potential inhibition by gossypol, which binds to the BH3 binding groove of Bcl-2 family members, warrant further investigation. Indeed, BH3 peptides have been shown to induce mitochondrial fission independent of Bax/Bak-dependent apoptosis signalling (Shroff et al., 2009).

We also implemented a GBM xenograft model to test the combination of gossypol and TMZ in vivo. Significantly reduced tumour burden was observed in animals treated with combination gossypol/TMZ (after 2 weeks), when compared with single-agent gossypol therapy. However, no significant reduction in tumour volume was evident for combination gossypol therapy vs treatment with TMZ alone. Nevertheless, we observed a reduced number of animals presenting with visible tumour necrosis in the treatment groups, particularly in the gossypol/TMZ-combined cohort (Figure 5D). This observation may indicate that tumour cell proliferation (as evident via Ki67 staining) is decreased following combined treatment leading to slower tumour growth and reduced tumour core necrosis. As previously reported (Soling et al., 2004; Heikkila et al., 2010; Goldman et al., 2011), we also observed extensive tumour necrosis in control tumours (Figure 5C) likely due to the rapid proliferation of U87MG-luc2 GBM cells when delivered SQ. Histopathological analysis showed that tumours from gossypol-treated animals have a similar histological architecture to control tumours. Furthermore, combination therapy does not manifest as a histologically distinct phenotype when tumours are compared with those from the single-agent TMZ treatment group. Moreover, tumours treated with either a single or combined regimen had decreased numbers of mitotic figures (Figure 5E). This indicates that tumour cell division is inhibited by gossypol and/or TMZ and is in line with other reports (Wolter et al., 2006; Filippi-Chiela et al., 2013; Shen et al., 2014). Examining more closely viable tumour histology in the non-necrotic regions, a significant decrease in proliferation was observed following combined gossypol/TMZ treatment as determined by Ki-67 staining, whereas a significant increase in the percentage of apoptotic cells was seen in the gossypol/TMZ combination treatment group as determined by TUNEL staining (Figure 5E, F and G). This suggests that the activity of gossypol and TMZ combined elicits cytotoxic and anti-proliferative effects in vivo. PECAM-1 staining of tumour tissue sections revealed a decrease in MVD (Figure 5E and H) underscoring the anti-angiogenic response of combined treatment observed in vitro (Figure 3).

In order to investigate combined gossypol/TMZ therapy in more clinically relevant patient-derived models and taking into account GBM heterogeneity, we examined treatment effects in three primary GBM cultures: patient-derived multicellular spheroids (Jarzabek et al., 2013a) and neurospheres derived from two patients with differing MGMT clinical status (BT224-luc2—methylated MGMT and BT248-luc2— unmethylated MGMT). We observed greater resistance to TMZ treatment in patient-derived multicellular spheroids and neurosphere cultures when compared with adherent cell lines. Nevertheless, combined treatment enhanced primary GBM cell death when compared with TMZ alone.Extent of response varied for individual patients tested (Figure 6) likely due to differences in tumour molecular fingerprints. The genetic profiles of primary GBM biopsy and in vivo multicellular spheroids showed typical GBM aberrations, such as: homozygous deletion of the PIK3R1 gene on chromosome 5; amplification of chromosome 7 including EGFR; homozygous deletion of CDKN2A/B and loss of one copy of chromosome 9; and homozygous deletion of chromosome 10 including the PTEN gene as described previously (Keunen et al., 2011) (Figure 6A, Supplementary Table S1). Among neurosphere lines (Figure 6B and C), we observed a greater decrease in cell viability in BT224-luc2 following gossypol alone and gossypol/TMZ combined treatment. This line, in contrast to BT248-luc2, showed methylated MGMT clinical status and no homozygous deletion of CDKN2A/B (Supplementary Table S1).

In summary, data presented herein indicate that implementing of a gossypol/TMZ combined regimen may be of relevance in the context of GBM. As presented, gossypol when combined with TMZ targets multiple ‘hallmarks of cancer’ (tumour proliferation, cell death and angiogenesis), which may provide benefit in patients presenting with advanced malignancies. Nevertheless, the extent of synergy observed is likely to be patient dependent. Mcl-1 and MGMT status may effect treatment outcome. Our data support further investigation of gossypol/TMZ combined approach in clinically relevant orthotopic models and ultimately in human trials.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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