CHARACTERISATION OF THE EFFECTS OF CHRONIC ASPIRIN TREATMENT ON THE VIABILITY AND PROLIFERATION OF STAGE 4 GLIOBLASTOMA CELLS

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Abstract – Epidemiological and experimental evidence exists to suggest that aspirin may possess anti-cancer properties in brain cancer, including glioblastoma multiforme (GBM). The aim of this study was to evaluate the effect of clinically relevant aspirin concentrations on the viability and proliferation of glioblastoma cells in order to establish the role aspirin may have as a potential therapeutic/preventative agent in GBM. PrestoBlue reagent was used to determine the viability of U-87 MG glioblastoma cells and SVG p12 astroglia cells over 7 days following aspirin treatment. CFDA-SE reagent was used to determine the effect of aspirin on U-87 MG cell proliferation over 72 hours. Aspirin did not appear to have a statistically significant effect on U-87 MG or SVG p12 cell viability over 7 days, although trends of reduced viability were seen at day 5 and 7 post drug treatment. Aspirin did not have an inhibitory effect on U-87 MG cell proliferation at 24 and 48 hours post drug treatment, although a slight effect of reduced proliferation was seen at 72 hours post drug treatment. Although further clinically relevant research still needs to take place, along with confirming aspirin's anti-cancer mechanisms, it is likely that low dose, long term aspirin use could have a future role in preventing GBM, or as an adjuvant therapeutic agent in GBM.

Keywords – Cancer, Glioblastoma Multiforme, Aspirin, Brain Tumour, Glioma, Proliferation, Viability.

Introduction

Glioma (tumours arising from glial cells) are the most common primary brain tumour, with approximately 1,860 new cases in England and Wales each year (NICE 2007). Glioblastoma multiforme (GBM) [World Health Organization (WHO) grade IV astrocytoma] is the most common and most aggressive of all glioma. According to NICE guidelines (2007), treatment for GBM usually involves surgical resection if possible, followed by radiotherapy. Temozolomide (an oral alkylating agent) can also be used for the treatment of newly diagnosed GBM. However, despite recent advances in cancer therapy, there has been little improvement to clinical outcome, as median survival time following diagnosis has only increased from 5 to 7 months in the last 20 years (Macmillan 2011). It is therefore essential that further research is conducted in order to understand the condition and discover safe and effective therapeutic and preventative agents to improve the dismal prognosis.

Aspirin was first synthesized in 1898 by Felix Hoffmann, and was initially used as an analgesic, antipyretic and anti-inflammatory agent. Meta-analyses of many randomized controlled trials have supported the use of regular low dose aspirin in the prevention of cardiovascular events (e.g. myocardial infarction) in patients at high risk, through its anti-platelet mechanism (Baigent et al. 2009).

Recently, epidemiological and experimental evidence suggested that long term, low dose aspirin use is associated with a reduced risk of colorectal cancer, with more limited evidence suggesting that there could also be an association between aspirin use and a decreased risk of brain cancer, including GBM (Sivak-Sears et al. 2004; Rothwell et al. 2011). Although the mechanism of how aspirin may produce an anti-cancer effect is still poorly understood, both cyclooxygenase (COX) dependent and independent mechanisms have been implicated.

Previous *in vitro* studies have shown that aspirin can display anti-proliferative effects on U-87 MG and T98G glioblastoma cell lines when used in the millimolar concentration range over 72 hours (Amin et al. 2003; Lan et al. 2011). These concentrations are likely to be unachievable at the site of action *in vivo* without causing severe side effects such as gastrointestinal bleeding and renal failure (Chyka et al. 2007).

The aim of this study was to evaluate the effect of clinically relevant aspirin concentrations (in the micromolar range) on the viability and proliferation of U-87 MG cells over an extended period of time (7 days), in order to establish the role that aspirin may have as a potential therapeutic/preventative agent in GBM. Taking into account the evidence presented from available literature, it was hypothesised that aspirin would reduce the viability and proliferation of stage 4 glioblastoma cells in a dose and time dependent manner.

Materials and methods

Chemicals

Aspirin and cisplatin were obtained from Sigma. PrestoBlue and CFDA-SE were obtained from Life Technologies (Invitrogen). All other chemicals were of the highest quality available and purchased from Sigma.

Maintenance of Cell Line

The human glioblastoma cell line U-87 MG and the human embryonic cell line SVG p12 were

obtained from the American Type Culture Collection (ATCC). The cells were grown and maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids and the antibiotics streptomycin (25 μg/ml) and penicillin (25 units/ml). Cells were kept at a temperature of 37 ˚C in an atmosphere of 5% CO_2 and were subcultured at 80% confluency using a 1:4 split ratio.

Cell Viability Assay

The viability of U-87 MG and SVG p12 cells was assessed using the PrestoBlue assay. The cells were seeded in three 96-well plates at a density of 100 cells per well in a volume of 100 μl and were allowed to adhere overnight. The cells were then treated with aspirin to produce a final concentration of 1, 3, 10, 30 and 100 μM and subsequently incubated for up to seven days. PrestoBlue reagent was added to a single plate at day 3, 5 and 7 post drug treatment, and was read using a plate reader at an excitation wavelength of 535nm and emission wavelength of 612 nm. Experiments were repeated three times and treatments were performed in triplicate within each experiment. A control drug (cisplatin, at a final concentration of 10 and 100 μM) was used, in addition to the control cell line (SVG p12).

Cell Proliferation Assay

Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was used to assess the effect of aspirin on U-87 MG cell proliferation. The cells were seeded in 6-well plates at a density of 150 000 cells per well in a total volume of 2 ml and were allowed to adhere overnight. CFDA–SE solution at a final concentration of 5 μM was added to the appropriate wells and incubated at 37˚C for 30 minutes. The labelling reaction was stopped by removing the CFDA-SE and adding fresh media. The cells were treated with aspirin at a final concentration of 10 and 100 μM and harvested at 24, 48 and 72 hours post drug treatment. Following harvesting, cells were pelleted by centrifugation, and were fixed at room temperature using 4% paraformaldehyde. The cells were re-suspended in 100 μl of PBS and fluorescence analysed using flow cytometry. A control drug (cisplatin, at a final concentration of 10μM) was also used in the assay.

Statistical Analysis

Commercially available software (GraphPad Prism) was used for data analysis, establishing IC_{50}. A two-way ANOVA test was used to analyse the significance between groups. The level of significance was set at $p < 0.05$.

Results

Effect of increasing cell number on the viability of U-87 MG cells over 7 days

Fig. 1 shows there is an increase in RFU (relative fluorescence units) with increasing cell number. Additionally, there is an increase in RFU for each cell density over time. An appropriate cell density for subsequent drug treatment viability assays would be 100 cells per well.

(a)
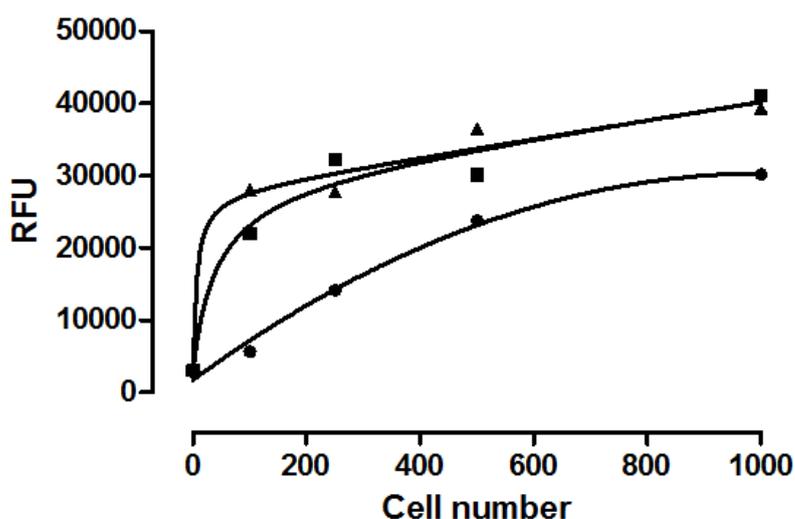
Figure 1: Graph showing the relationship between viability (relative fluorescence units) of U-87 MG cells and increasing cell number at day 3 (━◆━), 5 (━■━) and 7 (━▲━) after seeding. Data represent the average of four replicates for a single experiment. An appropriate cell density for subsequent drug treatment viability assays would be 100 cells per well.
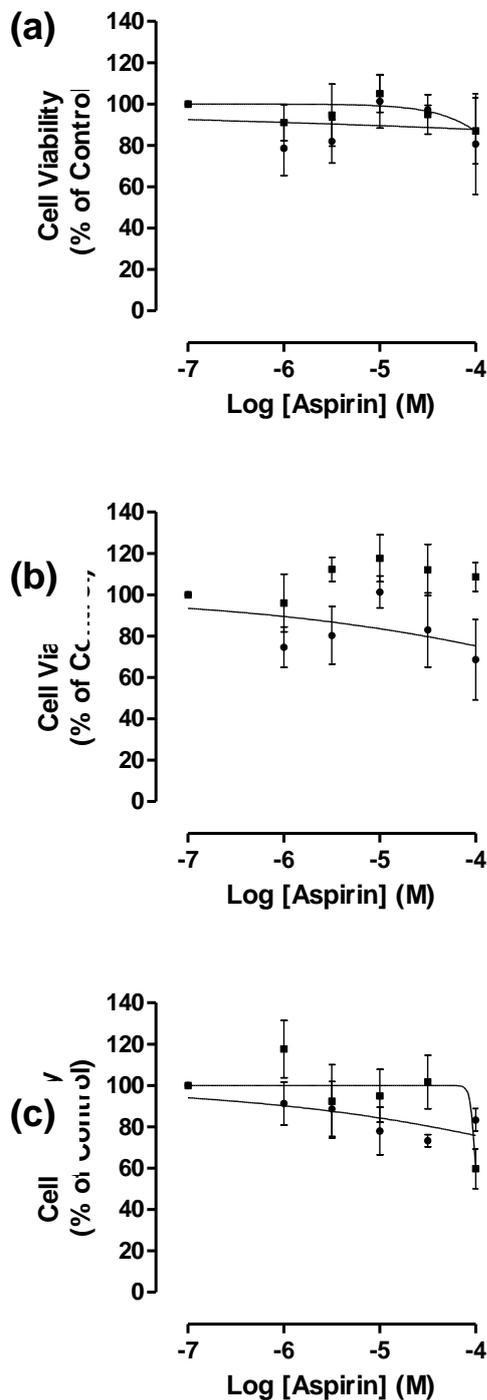
Effect of aspirin on the viability of U-87 MG cells compared with SVG p12 cells

As shown in Fig. 2, no statistically significant difference in cell viability was found between U-87 MG and SVG p12 cells following aspirin treatment (1-100 µM) at day 3 and 7 ($p > 0.05$). However at day 5 a statistically significant difference was observed, showing that aspirin decreased the viability of U-87 MG cells to a greater extent than the control cell line, SVG p12 ($p < 0.05$).
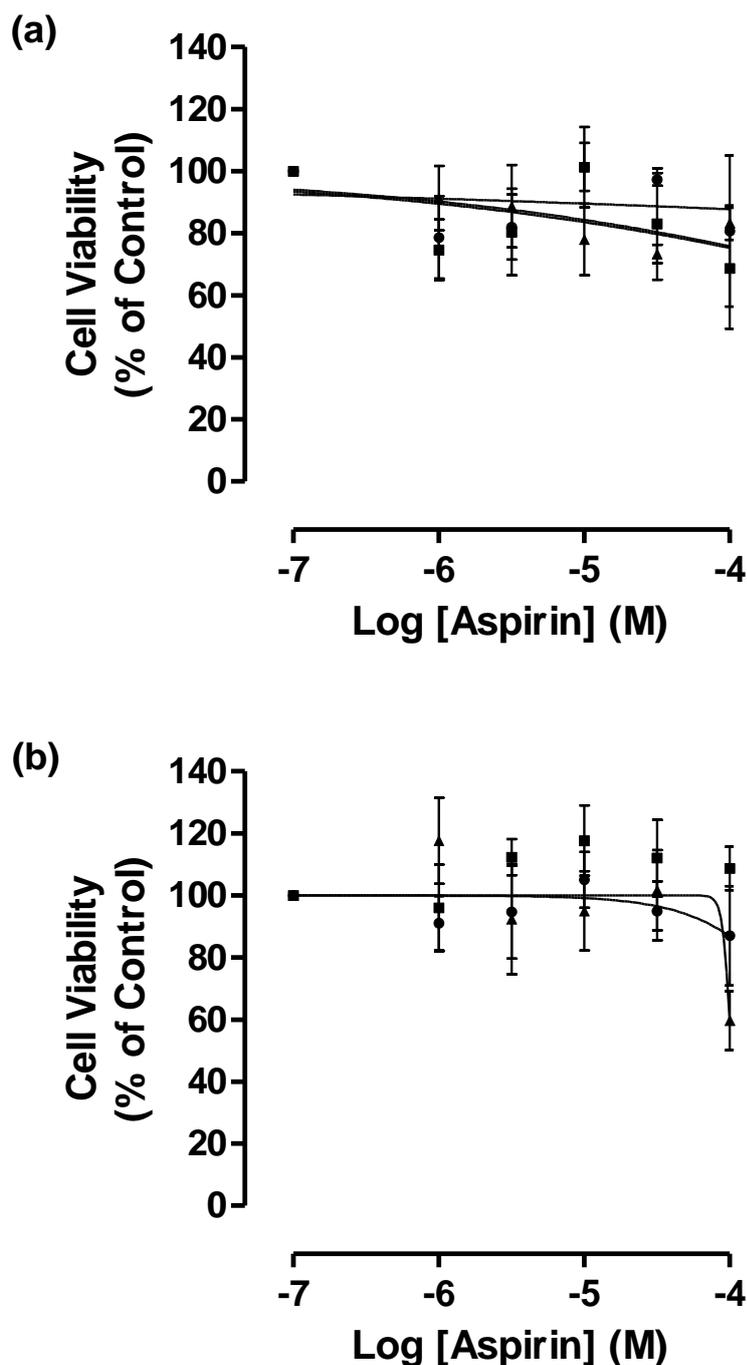
Effect of aspirin on the viability of U-87 MG and SVG p12 cells over time

Aspirin did not display a statistically significant dose or time dependent decrease in U-87 MG or SVG p12 cell viability, as shown in Fig. 3 ($p > 0.05$).Overall, trends were observed at days 5 and 7 that showed higher concentrations of aspirin led to a decrease in U-87 MG cell viability. For example, aspirin 100 µM decreased cell viability by 31% at day 5 compared with 19% at day 3. Lower concentrations of aspirin did not appear to have the same effect, with aspirin 1 µM showing only a

9% decrease in cell viability at day 7. An accurate IC_{50} could not be established from the data as aspirin did not inhibit viability to 50% or less.

Figure 2: Graphs showing the effect of aspirin (1, 3, 10, 30 & 100 µM) on the viability of U-87 MG cells (━●━) compared with SVG p12 cells (━■━), as determined by PrestoBlue assay. **(a):** day 3 post drug treatment (p > 0.05; two-way ANOVA), **(b):** day 5 post drug treatment (p < 0.05; two-way ANOVA), **(c):** day 7 post drug treatment (p > 0.05; two-way ANOVA). Data represent the mean of three independent experiments.
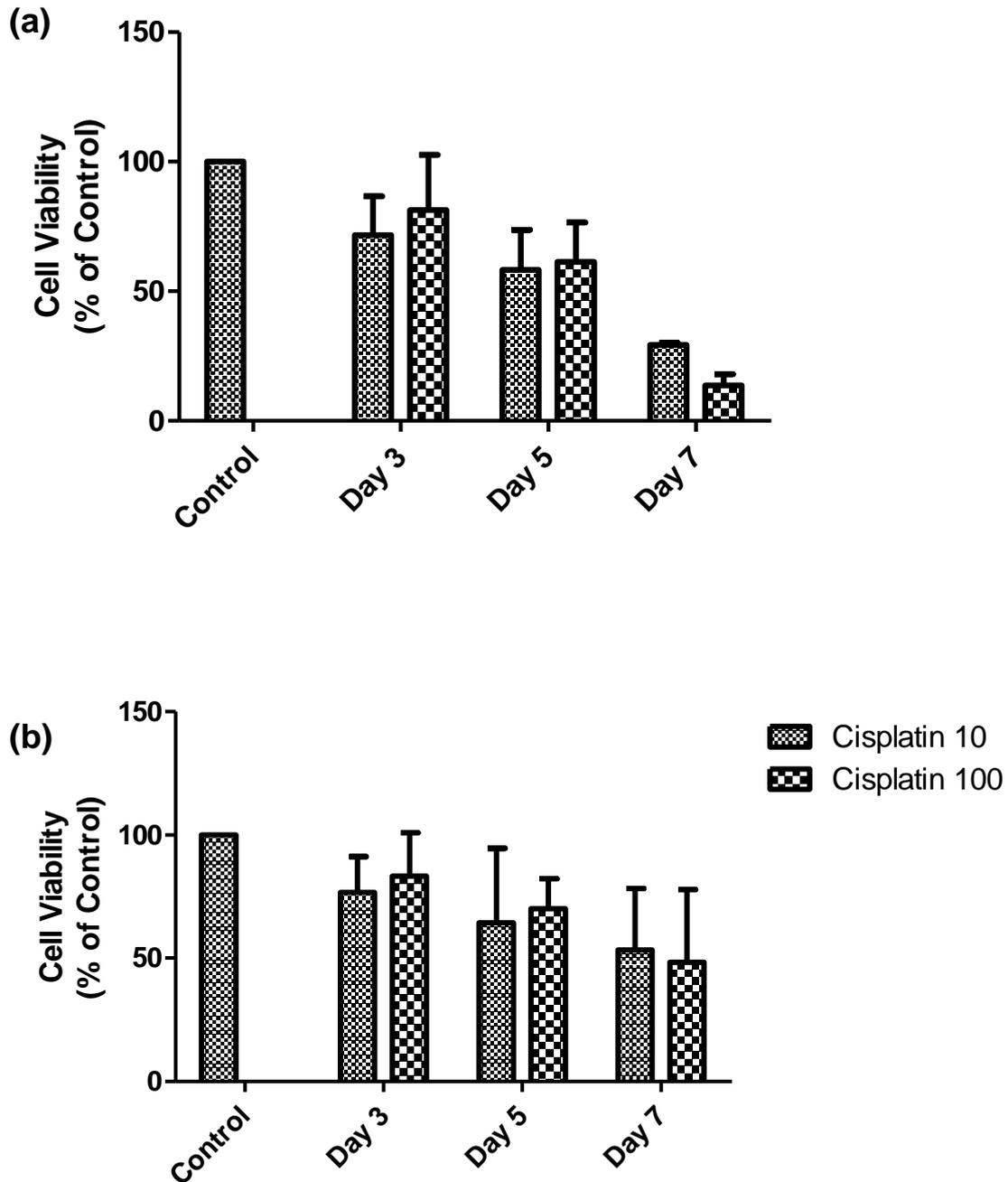
Figure 3: Graphs showing the effect of aspirin (1, 3, 10, 30 & 100 µM) on the viability of **(a):** U-87 MG cells and **(b):** SVG p12 cells at day 3 (━●━), day 5 (━■━) and day 7(━▲━) post drug treatment. No significant effect on cell viability over time was observed ($p > 0.05$; two-way ANOVA). Data represent the mean of three independent experiments.

Effect of cisplatin on the viability of U-87 MG and SVG p12 cell over 7 days

Cisplatin treatment at a concentration of 10 and 100 µM decreased the viability of both U-87 MG and SVG p12 cells in a time dependent manner (Fig. 4), and this effect was statistically significant in

U-87 MG cells ($p < 0.05$). The effect was particularly pronounced at day 7, where cisplatin 10 and 100 µM caused a 71% and 86% decrease in cell viability in U-87 MG cells respectively, and a 47% and 52% decrease in SVG p12 cells.
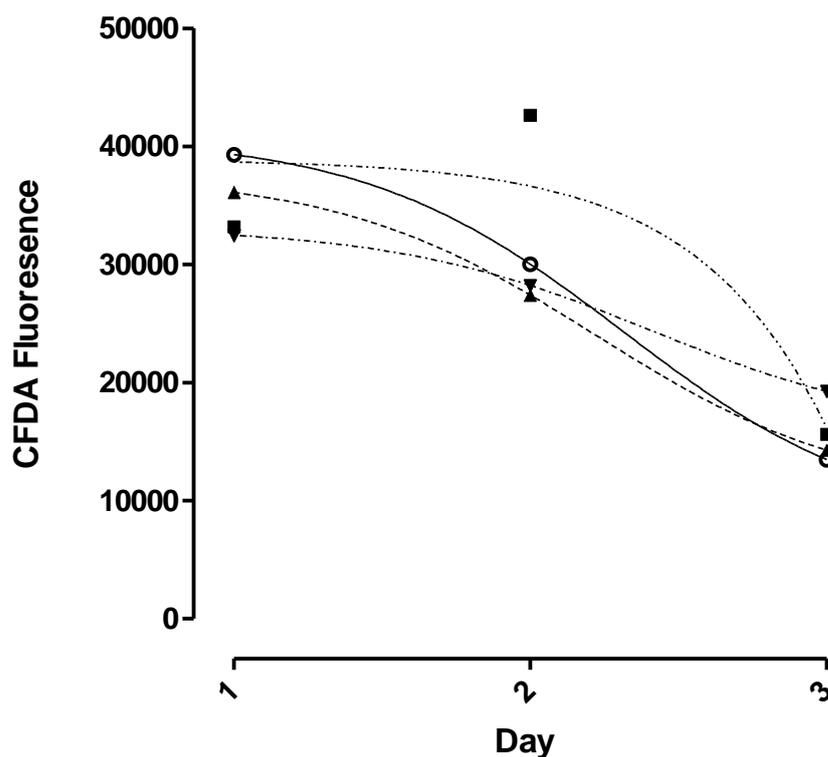

Figure 4: Graphs showing the effect of cisplatin 10 µM (▨) and 100 µM (▨) on the viability of **(a):** U-87 MG cells and **(b):** SVG p12 cells at day 3, 5 and 7 post drug treatment, as determined by PrestoBlue assay. A significant effect of time was found in U-87 MG cells following cisplatin treatment ($p < 0.05$; two-way ANOVA). Data represent the mean of three independent experiments.

Effect of aspirin on the proliferation of U-87 MG cells over 72 hours

Fig. 5 shows that aspirin (both 10 and 100 µM) did not inhibit the proliferation of U-87 MG cells at 24 or 48 hours, when compared with control conditions (no treatment, CFDA-SE stained). However a slight inhibition of proliferation was seen at 72 hours with aspirin 100 µM (fluorescence reading of 14287 compared with the control reading of 13479). Aspirin 10 µM also displayed anti-proliferative effects at 72 hours, to a greater extent than aspirin 100 µM (fluorescence reading of 19253 compared with control reading of 13479).

Effect of cisplatin on the proliferation of U-87 MG cells over 72 hours

Cisplatin 10 µM displayed anti-proliferative effects on U-87 MG cells at 48 and 72 hours post drug treatment (Fig. 5). At 48 hours, cisplatin had a fluorescence reading of 42640 compared with control reading of 30037. At 72 hours, cisplatin had a fluorescence reading of 15629 compared with control reading of 13479.

Figure 5: Graph showing the effect of aspirin 10 µM (▼), 100 µM (▲) and cisplatin 10 µM (■) on the proliferation of U-87 MG cells at day 1, 2 and 3 post drug treatment, compared with control condition (no treatment, CFDA-SE stained) (—O—).CFDA fluorescence measured by flow cytometry indicated proliferation, with cell division resulting in sequential halving of the initial fluorescence. Data represent results from a single experiment.

Discussion

The present study was undertaken to evaluate the effect of clinically relevant aspirin concentrations on the viability and proliferation of U-87 MG glioblastoma cells. Taking into account available literature, it was hypothesized that aspirin (1-100 μM) would decrease the viability and proliferation of stage 4 glioblastoma cells in a dose and time dependent manner. However, the hypothesis was not supported by the data obtained, as aspirin appeared to have no statistically significant effect on the viability and proliferation of U-87 MG cells over time, although certain trends were observed.

Aspirin inhibits the COX isoenzymes; COX-1 and COX-2 (Simmons et al. 2004). COX-1 is present in nearly all cell types at a constant level. Inhibition of COX-1 in platelets leads to decreased synthesis of thromboxane A2 (TA2), which results in decreased platelet aggregation. Inhibition of COX-1 results in decreased production of the protective stomach and kidney prostaglandins, and this is responsible for the adverse gastrointestinal and renal effects of aspirin (Vane et al. 1998).

COX-2 is normally absent, but is inducible in response to cytokines, interleukin-2 (IL-2) and tumour necrosis factor (TNF-α). The prostaglandins produced *via* the COX-2 pathway are therefore associated with inflammation and inhibition of this pathway is responsible for aspirin's anti-inflammatory action (Vane et al. 1998). Selective COX-2 inhibitors (e.g. Rofecoxib) were developed in order to have improved gastrointestinal toxicity profiles. These agents were initially evaluated for their potential anti-cancer properties until safety concerns about cardiovascular toxicity were raised in 2004 (Bresalier et al. 2005).

Shono et al. (2001) showed that COX -2 is over expressed in glioma, particularly GBM, leading to increased production of prostaglandins. COX-2 prostaglandins have been shown to promote the formation of tumours by inhibiting apoptosis, modulating the immune system and regulating tumour angiogenesis (Cha and Dubois 2007). Baryawno et al. (2008) also suggested that enhanced prostaglandin synthesis can directly stimulate cell growth in brain tumours. It would therefore be expected that aspirin would have a greater inhibitory effect on glioma cell growth as opposed to non-cancerous astroglia cells. This could explain why the day 5 result of this study (Fig. 2), showed that aspirin decreased the viability of the U-87 MG glioblastoma cell line to a greater extent than the astroglia control cell line; SVG p12.

COX independent mechanisms accounting for aspirin's anti-cancer effects have also been proposed. These have attracted much attention since non-steroidal anti-inflammatory drugs (NSAIDs) that do

not inhibit COX still inhibit tumour formation in mice models (Piazzaet al.1997). Furthermore, more potent inhibitors of COX do not necessarily show greater inhibitory effects on tumours. *In vitro* and *in vivo* evidence has suggested aspirin may inhibit the activation of nuclear factor κB (NFκB) (Stark et al. 2007). When activated, NFκB turns on the expression of genes that keep cells proliferating. Furthermore, *in vitro* evidence has demonstrated that aspirin can interfere with β-catenin and wnt signalling (Lan et al. 2011; Bos et al. 2006). The wnt signalling pathway increases the level of the transcription factor Myc, which in turn increases the transcription of cyclin D. Cyclin D acts on retinoblastoma protein to activate the formation of cyclin A and cyclin E, with the latter being responsible for driving the cell into the cell cycle (Berridge 2012). Studies have also suggested that aspirin can interfere with the cell cycle by inducing the degradation of cyclin D1 (Alao 2007).

In the present study, aspirin at concentrations ranging from 1-100 μM did not appear to have a statistically significant effect on reducing U-87 MG cell viability or proliferation. Only one set of data was generated from the proliferation assay, therefore it would need to be repeated (to n=3) to determine if consistent results can be obtained. Similar effects were deduced by Amin et al., (2003), who found that aspirin at concentrations less than 500 μM did not show statistically significant growth inhibitory effects on T98G glioblastoma cells. Furthermore, Borthwick et.al. (2006) found that aspirin 500 μM (equivalent to a low therapeutic plasma concentration) did not result in a significant decrease in endothelial HMEC-1 cell viability or proliferation. However, this dose did lead to a significantly decreased ability of the cells to undergo angiogenesis (as measured through a 3D collagen angiogenesis assay).

This finding is of significance, as it could therefore suggest that low dose aspirin concentrations are producing an inhibitory effect on glioma cells *in vivo* due to a decrease in angiogenesis. Angiogenesis may not be accounted for in viability and proliferation experiments *in vitro*, and hence could be the reason why little or no effect on glioma cell viability and proliferation has been shown with the low aspirin concentrations used in this study, despite epidemiological and experimental evidence supporting the use of low dose aspirin (Sivak-Sears et al. 2004; Rothwell et al. 2011). An extension of the present study could therefore involve examining angiogenesis using the same aspirin concentrations (1-100 μM) to determine efficacy.

Hwang et al. (2004) showed that low dose aspirin is as effective as high dose aspirin in reducing prostaglandin E2 synthesis through COX-2 inhibition in glioma cells. If COX dependent mechanisms were entirely involved in aspirin's anti-cancer mechanism, then it would be likely that both low and

high dose aspirin would have similar effects on glioma viability and proliferation, which has been shown not to be the case. High aspirin concentrations may therefore produce an effect through COX independent mechanisms such as interfering with β-catenin and wnt signalling (Lan et al. 2011; Bos et al. 2006). The idea that different concentrations of aspirin produce anti-cancer effects via different mechanisms was also proposed by Arrieta et al. (2001).

In the present study, trends of reduced cell viability were observed at day 5 and 7 (Fig. 3), although overall there was a large degree of variability. This variability may be attributed to the use of a haemocytometer for cell counting. It has been well documented that the use of a haemocytometer for cell counting can lead to inaccuracies and subjectivity (Hefner et al. 2010); which could potentially impact on the viability and proliferation data generated, as a result of inconsistent plate seeding. The cell counting procedure could be improved by using an automated cell counter such as the TC10™ which could lead to more accurate and reproducible results. Furthermore, the viability assay could be repeated and/or extended to 10 days to see if the trends observed turn into statistical significance.

In relation to non-statistically significant trends observed in the data, possible reasons for aspirin having more effect on viability at day 5 and 7 could be because *in vitro* conversion of aspirin to salicylate (an active metabolite of aspirin) takes place over time. Salicylate may have a more prominent anti-cancer effect than aspirin. However, since salicylate is a poor inhibitor of COX compared with aspirin, it could further allude to a COX independent anti-cancer mechanism, such as the ability of salicylate to directly activate AMP-activated protein kinase (AMPK) (Hawley et al. 2012). Interestingly, temozolomide, a first line chemotherapeutic agent in GBM, has been found to contribute to apoptosis in glioblastoma cells through activation of AMPK (Zhang et al. 2010).

The fact that the data trends observed show that aspirin decreases U-87 MG cell viability to a greater extent following extended exposure (day 5 and 7), could suggest long-term, low dose aspirin use may have a role as a preventative agent in GBM.

Cisplatin proved to be very efficient at decreasing the viability of both U-87 MG and SVG p12 cells, particularly at day 7 post drug treatment. Cisplatin also reduced the proliferation of U-87 MG cells at 48 and 72 hours post drug treatment. This is as expected, as cisplatin is a cytotoxic agent that interferes with normal transcription and DNA replication mechanisms (Fuertes et al. 2003). This significant result could provide a basis for researching future co-therapies involving aspirin and low

dose cisplatin, which could lead to an improved therapeutic effect without the toxicity associated with higher doses. For example, Ashktorab (2005) found that aspirin in combination with low dose 5-fluorouracil demonstrated a synergistic anti-cancer effect in colorectal cancer.

Since previous *in vitro* studies have shown high concentrations of aspirin display inhibitory effects on the growth of glioma cells (Amin et al. 2003; Lan et al. 2011), future research should also be committed to developing ways of promoting efficacious aspirin concentrations at the site of action *in vivo* whilst minimising systemic toxicity. This could be achieved through nanotechnology, which would enable glioblastoma cell targeting (Jarboe et al. 2007). Furthermore, the synthesis of novel analogues of aspirin with similar potency but reduced side effect profiles may also be an important future research strategy (Deb et al. 2011).

Conclusion

Whilst little progress has been made recently in improving the outcome for patients with GBM, research involving aspirin and GBM has produced some promising leads. Although further clinically relevant research still needs to take place, along with confirming aspirin's anti-cancer mechanisms, it is likely that low dose, long term aspirin use could have a future role in preventing GBM, or as an adjuvant therapeutic agent in GBM.

Acknowledgement

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