P-glycoprotein Blockers Augment the Effect of Mitomycin C on Human Tenon's Fibroblasts

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Purpose: Mitomycin C (MMC), which induces apoptosis in human Tenon's fibroblasts (HTF), is frequently used to retard wound healing after glaucoma surgery. The aim of this in vitro study was to examine whether adjunctive Verapamil and Cyclosporine could augment the cytotoxic effect of MMC on HTF.

Methods: Fibroblast cell lines were established by explant culture from human tissue biopsy samples obtained during trabeculectomy procedures. Cells were exposed to MMC at varying concentrations (0.01–0.4 mg/ml) for 3 minutes, prior to washing in the presence or absence of the following drugs: Staurosporine (0.003mg/ml), Verapamil (2.5–0.25 mg/ml), or Cyclosporine (50–0.5 mg/ml). Following exposure, cells were cultured for 6 hours and surviving cells quantitated by haemocytometer counts.

Results: Both Verapamil and Staurosporine exhibited mild toxic effects on their own, but greatly enhanced the apoptotic effect of MMC. Staurosporine is too toxic to be considered clinically, so its augmentive effect on the activity of MMC was not studied further here. Doses as low as 0.25 mg/ml of Verapamil continued to show significant augmentation of the apoptotic effect of MMC. Cyclosporine at a clinically used concentration (5 mg/ml) exhibited modest augmentation of the effect of MMC.

Conclusions: Verapamil and Cyclosporine in clinically acceptable concentrations potentiate the effect of MMC and may obviate the need for high dose antimetabolites in trabeculectomy; however, further preclinical study is required.

Translational Relevance: Adjunctive Verapamil or Cyclosporine may allow lower dose MMC to be used in glaucoma filtration surgery while maintaining the same antifibrotic effects.

Introduction

Glaucoma is the second most common cause of blindness in the world and the leading cause of irreversible blindness.¹ By 2020, it is estimated that there will be almost 80 million people with glaucoma, of whom, approximately 11 million will be blind.² The mainstay of treatment is intraocular pressure control, which can be achieved by medications, laser, or filtration surgery. Typically, medical therapy is the first line treatment and is suitable for the majority of patients. It is an ever evolving field.³ Surgery is

typically reserved for more aggressive glaucomas, and where medical therapy fails. Increasingly, guidelines for the treatment pathway are being developed and published though uniform adherence to them can be variable.⁴

The alkylating agent Mitomycin C (MMC) is the most widely used adjunct to glaucoma filtration surgery.⁵ Application of MMC induces apoptosis in human Tenon's fibroblasts (HTF), which slows wound healing and preserves subconjunctival aqueous drainage.⁶ However, Mitomycin toxicity can cause serious complications due to excessive wound

inhibition or collateral tissue damage.⁵ Antifibrosis treatment strategies that reduce the dose of MMC required, or better localize its effects have the potential to maintain effectiveness while reducing complications.

The role of the adenosine triphosphate (ATP)-dependent efflux pump P-glycoprotein (P-gp) in chemotherapeutic drug resistance is very well described.⁷ It is a member of the ATP-binding cassette superfamily and plays a role in removing toxic substances from within cells. Recently, P-gp expression has been shown to be upregulated in induced MMC resistance in cultured HTF, while this is overcome by blockade of the channel.⁸

Verapamil is the classically described P-gp inhibitor. Its inhibitory activity is believed to be as a competitive substrate rather than via its usual clinical application as a calcium channel blocking agent.⁹ Verapamil's role in overcoming chemotherapeutic agent resistance has been described for over 30 years.¹⁰ Induced MMC resistance in HTF can be overcome in vitro by utilizing the anti P-gp activity of Verapamil.⁸ Cyclosporine A has also been tried as an adjunct to glaucoma filtration surgery.¹¹ This agent is also commercially available at a low dose as an antidry eye medication (0.05% Restasis; Allergan, Irvine, CA). Cyclosporine and Verapamil are believed to have differing mechanisms of action in their inhibition of the activity of P-gp with Verapamil exhibiting a more ATP-dependent mechanism of action.¹²

The combined effect of interferon α and γ in augmenting the effect of MMC in inducing apoptosis has been previously described.¹² This activity is believed to be mediated by upregulation of components of the Fas-dependent apoptotic pathway rather than the activity of P-gp.¹³ Staurosporine is a well described broad spectrum protein kinase C inhibitor, which both reduces P-gp activity¹⁴ and induces cell death in a caspase independent manner.¹⁵ Staurosporine has been used at a concentration of 0.0003 mg/mL as an adjunct to MMC treatment in a rabbit experimental model of glaucoma surgery,¹⁶ but is too toxic to be considered for clinical use. For these reasons, we investigated the effect of Verapamil and Cyclosporine A, both known to inhibit P-gp activity, to see if they could augment the effect of MMC in inducing fibroblast death. We used a model utilizing cultured HTFs obtained from a small number of clinical samples, described previously.^{6,13,17} We compared this against the activity of Staurosporine and interferons α and γ .

Methods

Isolation and Culture of HTF

A HTF cell line was obtained from human biopsy specimens during routine cataract surgery at Westmead Hospital (New South Wales, Australia). Written consent was obtained from all patients. The tenets of the Declaration of Helsinki were observed, and approval of the Westmead Hospital human research ethics committee was granted. Inferior bulbar conjunctival biopsies were taken from seven patients between January 2004 and October 2005. None of the patients had been exposed to antimetabolites prior to sampling. Samples were collected in sterile jars containing Hanks Balanced Salt Solution (HBSS; Sigma, St. Louis, MO) supplemented with Penicillin (100 U/mL; Invitrogen, Carlsbad, CA), Streptomycin (100 ug/mL; Invitrogen), and Amphotericin B (2.5 ug/mL; Invitrogen). After extensively washing the biopsy tissue in HBSS, the specimens were cut into 1- to 2-mm pieces. Six-well tissue culture plates were scored with parallel lines using a scalpel in order to assist in tissue attachment. Pieces of dissected tissue were gently covered using a few drops of media consisting of Roswell Park Memorial Institute medium (RPMI; Invitrogen) supplemented with 20% Bovine Calf Serum (BCS; Bovogen, Essendon, Australia) and antibiotics. Tissues were then incubated at 37°C under 5% CO₂, and media was changed every 3 days. Confluent cells were released using trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA; 0.02%) in HBSS, and further cultured in gelatine-coated (0.1% in phosphate buffer solution [PBS; Kyowa Hakko Kogyo Ltd., Tokyo, Japan]) 75 cm² flasks in RPMI with antibiotics and BCS (10%). Cells were propagated at a ratio of 1:3 up to a maximum of eighth passage and stored frozen in liquid nitrogen.

Stimulation of Cells in Experiments

Cells were seeded at confluence in 500 μ L of full growth medium into gelatine-coated 24-well culture plates. After overnight culture, cells were pretreated with drugs known to have P-gp blocking activity for a period of 3 minutes being: Verapamil (0.25–2.5 mg/mL; Abbott, Sydney, Australia), Staurosporine (0.0003 mg/mL; Merck, Darmstadt, Germany), and Cyclosporine A (0.5–50 mg/mL; Novartis, Sydney, Australia). The dosages of interferon used were the same as in a previous study, being interferon α at 5000 U/mL and interferon γ at 100 U/mL (Merck).⁹ Cell death was induced by treating the fibroblast

monolayer with single applications of MMC reconstituted in PBS (Kyowa Hakko Kogyo Ltd.) for 3 minutes at varying concentrations consistent with those used in clinical practice in glaucoma filtration surgery. Experimental control was by pretreatment of cells with Verapamil, Cyclosporine A, interferon, or Staurosporine without subsequent exposure to MMC. Baseline cell count controls were treated with 3 minute applications of PBS only. After treatment, monolayers were washed three times with RPMI and further cultured in RPMI culture medium for 6 to 48 hours. The primary outcome measure was HTF cell survival and evaluated by cell count.

Evaluation of Cell Survival

Surviving adherent cells were quantified by hemocytometer cell counts. In brief, wells were washed twice with 500 μ L of RPMI and adherent cells then released using 100 μ L of trypsin with EDTA in HBSS. Suspended cells were diluted 1/10 in 0.4% trypan blue stain (Invitrogen), before loading approximately 10 μ L into each side of a Hirschmann hemocytometer (Hausser Scientific, Horsham, PA) and counting. Cells were trypsinized and counted at time periods of 6, 24, and 48 hours post treatment. A minimum of six experiments were carried out per condition and cell counts were replicated three times per experiment.

The possible role of apoptosis in experiments was evaluated by fluorescence activated cell sorting analysis (FACS) for annexin V-FITC binding apoptotic endothelium, combined with propidium iodide labelling DNA in cells with compromised plasma membranes as detailed in earlier work.⁹

Statistical analysis was carried out utilizing Statgraphics Online (Statpoint Technologies, Warrenton, Virginia) and graphical output was via Microsoft Excel (Microsoft, Redmond, WA). Comparison of multiple groups against a reference sample was by Bonferoni corrected Student's *t*-test. Comparison between multiple groups was by ANOVA *P* values of less than 0.05 were considered statistically significant, and Bonferoni correction for multiple comparisons was applied where appropriate.

Results

Mitomycin C Alone Reduced HTF Survival

HTF had the typical appearance of fibroblasts in culture (Figs. 1A, 1B), while experiments with MMC

were initially performed at a concentration of 0.4 mg/mL to mimic a treatment regimen typically used as an adjunct to glaucoma filtration surgery. Hours of MMC treatment resulted in an average of 42% reduction in HTF numbers and there was no statistically significant difference between treatment times ($P > 0.05$, Fig. 1C). In subsequent experiments, a 6 hour analysis post exposure to MMC was used.

Differing concentrations of MMC were next applied to establish a dose response for cell death. All doses used were in the range of clinical practice for glaucoma surgery, while the results are shown in Figure 1D. Control cells were present at approximately 2.5×10^5 cells per well. The effect of MMC was greatest at the highest concentration of 0.4 mg/mL, and approached control levels at 0.1 mg/mL. The three groups were statistically different (ANOVA $P < 0.001$). Apoptosis was confirmed in these cultures by FACS analysis for annexin-V and propidium iodide (data not shown). Using a Bonferoni corrected *t*-test, cell groups treated with 0.4 mg/mL and 0.2 mg/mL MMC achieved statistically significant reduction in cells with *P* values less than 0.001 counts, but the 0.1 mg/mL treated group did not ($P > 0.05$).

The Effect of Verapamil and Cyclosporine A on HTF Response to Mitomycin C

Verapamil at 2.5 mg/mL alone exerted, at best, a mild toxic effect on HTF with cells rapidly detaching from the culture surface, and this was very strongly potentiated by MMC treatment at all concentrations studied (*t*-test $P < 0.001$), while there were no statistically significant differences in this regard relating to the concentration of MMC (Fig. 2). Despite loss of HTF by detachment and subsequent apoptosis of detached cells, no clear effect of Verapamil upon HTF apoptosis, independent of detachment, was seen by FACS analysis (data not shown).

Treatment of HTF with 50 mg/mL of Cyclosporine A proved to be extremely toxic to the cells, such that only 22% of cells survived when treated with the Cyclosporine A alone, and HTF survival was further reduced to 8% when this high dose of Cyclosporine A was combined with 3 minutes of further treatment with 0.4 mg/mL of MMC. When Cyclosporine A was applied at a lower concentration of 5 mg/mL, HTF death was also observed ($P < 0.001$, Fig. 3), while there was also some modest augmentation of the

Figure 1. Photomicrographs comparing control HTF (A) with cells exposed to MMC at 0.4 mg/mL for 3 minutes followed by a further 24 hours incubation (B), as well as histograms showing the number of cells over time following such treatment (C), and the effect of varying concentrations of MMC (D). HTF had the characteristic fusiform appearance of fibroblasts in culture (A), and this was retained following treatment with MMC, although there did appear to be a reduction in cell number (B). Quantitation confirmed progressive reduction over 24 hours in HTF number following MMC treatment at 0.4 mg/mL (C), while lower concentrations of this agent had less effect on cell number (D). Error bars show SEM.

Figure 2. Photomicrographs of HTF treated with Verapamil (V) (2.5 mg/mL) for 3 minutes and then further incubated for 24 hours without (A) or with (B) MMC treatment for a further 3 minutes at 0.4 mg/mL, as well as quantitation of surviving cell number following such V pretreatment with subsequent 3 minute exposure to MMC ranging from 0 to 0.4 mg/mL (C). V treatment alone had little effect on the appearance or number of HTF (A), but when combined with further MMC treatment did significantly reduce cell number and alter the morphology of surviving cells (B). (C) This effect of V at greatly potentiating the effect of MMC was seen at all concentrations of MMC studied (t -test $P < 0.001$), while varying the concentration of MMC did not have any clear effect (ANOVA, $P > 0.05$). Error bars show SEM. Asterisks denote statistical significant result.

Figure 3. Photomicrographs of HTF treated Cyclosporine A (CA) (0.5 mg/mL) for 3 minutes and then further incubated for 24 hours without (A) or with (B) MMC treatment for a further 3 minutes at 0.4 mg/mL, as well as quantitation of surviving cell number following such CA pretreatment with subsequent 3 minute exposure to MMC ranging from 0 to 0.4 mg/mL (C). CA treatment alone had little effect on the appearance or number of HTF (A), but when combined with further MMC treatment did significantly reduce cell number and alter the morphology of surviving cells (B). (C) This effect of CA of greatly potentiating the effect of MMC was seen for MMC at 0.2 mg/mL and 0.4 mg/mL (t -test $P < 0.001$), but not at the lowest concentration tested. Error bars show SEM. Asterisks denote statistical significant result.

Figure 4. Histograms showing the number of surviving HTF relative to control cells following initial pretreatment for 3 minutes with either Staurosporine (S) (0.3 μ g/mL) (A), or Interferons α and combined (INF) at 500 and 100 U/mL respectively (B), followed in both cases by further incubation with MMC at concentrations ranging from 0 to 0.4 mg/mL. (A) Although S pretreatment did appear to have a modest effect in increasing sensitivity to MMC induced HTF death, this was only statistically significant when MMC was at a concentration of 0.2 mg/mL ($P = 0.01$, t -test). (B) Combined INF treatment significantly reduced HTF survival when cells were treated with MMC in a clearly dose dependent manner ($P < 0.001$). A three-way ANOVA test revealed a significant difference between treated groups ($P < 0.001$). Error bars show SEM. Asterisks denote statistical significant result.

Figure 5. The effect of varying the pretreatment concentration of V, expressed as a percentage of 2.5 mg/mL, on the HTF cell counts of cells treated with 0.4 mg/mL MMC. Incubation time post treatment was 6 hours. At concentrations as low as 0.25 mg/mL V there was still marked augmentation of the effect of MMC. The effect was not as marked at the lowest dose of V tested (0.125 mg/mL). Error bars show SEM. The effect of V was statistically significant across all groups ($P < 0.001$).

activity of MMC at 0.4 mg/mL and 0.2 mg/mL compared with Cyclosporine A alone ($P < 0.001$), although there was no effect on MMC at a concentration of 0.1 mg/mL (Fig. 3C).

The Effect of Staurosporine and Interferon on HTF Response to Mitomycin C

Treatment of HTF with Staurosporine alone resulted in a marked reduction in cell count ($P < 0.001$, Fig. 4A). Additional treatment with varying concentrations of MMC resulted in a further, modest reduction in HTF survival, but this was only statistically significant when MMC was at a concentration of 0.2 mg/mL ($P = 0.01$, *t*-test) (Fig. 4A). A three-way ANOVA test did not reveal a statistically significant difference between the three MMC treated groups. Pretreatment with interferons α and γ also showed augmentation of the effect of MMC in a dose-dependent manner ($P < 0.001$, Fig. 4B).

The Effect of Lower Dose Verapamil on the HTF Response to Mitomycin C

Figure 5 shows the effect of decreasing concentrations of Verapamil, the most effective of the three P-gp inhibitors tested, with and without MMC at 0.4 mg/mL. MMC-induced HTF death by Verapamil was strongly enhanced at all concentrations studied ($P < 0.001$), and this was only modestly reduced at the lowest concentration of Verapamil (0.125 mg/mL).

Discussion

Utilizing a well-established experimental model, this preliminary study indicates varying effect of P-gp inhibitors upon MMC-induced HTF survival, with Verapamil displaying the most promising potential clinical value.

The current findings support an earlier report demonstrating induction of MMC-mediated HTF death by Verapamil in MMC-resistant cells where the mechanism of resistance was via poor and prolonged exposure to low dose MMC.⁸ Our study shows that very low dose Verapamil used as a pre-rather than post-treatment, can augment the effect of MMC in HTF preparations that are not MMC resistant. Verapamil treatment alone at a concentration of 2.5 mg/mL does appear to be clinically safe in glaucoma filtration surgery although no clear clinical benefit is reported.¹⁶ The current study, however, suggests that use of Verapamil concomitant with

MMC may be clinically efficacious while permitting reduced levels of MMC to be used. A previous study in rabbits also suggested this.¹⁸ In addition, the action of Verapamil on rendering the effect of MMC on HTF cell death relatively dose insensitive, raises the possibility of low dose MMC being applied more precisely during surgery with less potential for complications in adjacent areas. The mechanism through which Verapamil enhances MMC-induced HTF death is unclear, and appears primarily related to enhanced detachment of the cells rather than enhanced MMC-induced HTF apoptosis.

The other drugs tested did not demonstrate the same potential as Verapamil, and so were not further studied. Cyclosporine A, a nominally 'safer' non-cardiogenic drug with known anti P-gp activity, did not prove a likely candidate for clinical utility in this context. Relatively high dose Cyclosporine A proved extremely toxic to HTF and low concentrations showed only modestly encouraging effects. This correlates with in vivo and clinical studies, which show that higher dose Cyclosporine A tends to induce significant dose-dependent fibroblast apoptosis in vivo,^{19,20} and encourages bleb failure in MMC augmented trabeculectomy in rabbits,²¹ but clinically has no impact on the function of antimetabolite augmented trabeculectomy at low dose.¹¹

Staurosporine is an extremely toxic drug in itself used for experimental purposes only, and so would be difficult to use clinically. It has broad spectrum antiprotein kinase C activity, which can affect the activity of ATP binding cassette proteins such as P-gp.²² In our experiments, it was difficult to separate the effects of Staurosporine from any truly synergistic activity with MMC. The main reason for inclusion in this study was to confirm a previous in vivo report¹⁸ of the effect of Staurosporine in augmenting the effect of MMC.

The effect of interferon α and γ on augmenting the apoptotic effect of MMC on HTF preparations via a Fas-dependent mechanism has been previously described.¹³ The relative insensitivity of the dose response to Verapamil in augmenting the effect of MMC as compared with that of combined interferon treatment offers potentially greater clinical utility for Verapamil, as it can be given at very low dose and still have effect.

Based on these preliminary findings, we believe further in vitro, in vivo, and clinical study is justified to investigate the potential benefit of Verapamil in MMC treatment during glaucoma surgery.

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