

## EXTENDED REPORT

## Effects of celecoxib in human retinoblastoma cell lines and in a transgenic murine model of retinoblastoma

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**Background/aim:** Celecoxib, a cyclooxygenase-2 inhibitor and antiangiogenic agent, has demonstrated potent anticancer effects in preclinical studies and in human clinical trials. To evaluate the potential utility of this agent in the treatment of retinoblastoma, the authors investigated the effects of celecoxib in retinoblastoma cell lines and in a murine model of this disease.

**Methods:** Growth inhibitory effects of celecoxib were evaluated in Y79 and Weri-RB1 human retinoblastoma cell lines by WST-1 cell proliferation assay. For animal study, two groups of 24, 8 week old LH $\beta$ -TAg transgenic mice were treated with celecoxib (250 mg/kg, orally once a day) or vehicle control, 5 days/week for 6 weeks. Mice were sacrificed on day 43. Enucleated eyes were serially sectioned and ocular tumour burden was quantified by histopathological analysis.

**Results:** Celecoxib did not inhibit proliferation of Y79 or Weri-RB1 cells, even at concentrations far exceeding clinically achievable levels. No significant difference in ocular tumour burden between celecoxib treated and control mice ( $p=0.73$ ) was found.

**Conclusion:** Celecoxib was ineffective at inhibiting proliferation of retinoblastoma cells in vitro and was ineffective at controlling retinoblastoma tumour growth in a murine model of this disease. On the basis of these findings, oral celecoxib therapy is unlikely to have clinical utility in the treatment of retinoblastoma.

Retinoblastoma is the most common paediatric primary ocular malignancy, representing 12% of infant cancers.<sup>1</sup> This tumour results from homozygous mutation of the retinoblastoma tumour suppressor gene (*RB1*) in retinal progenitor cells.<sup>2,3</sup> In non-heritable retinoblastoma, both mutations occur somatically in a single cell, resulting in unifocal, unilateral disease. In heritable retinoblastoma, one *RB1* allele is mutated in the germline and the second allele is inactivated somatically, usually resulting in multifocal, bilateral disease.

Until the last decade, higher stage intraocular retinoblastoma was conventionally treated with enucleation or external beam radiotherapy (EBRT). However, EBRT is associated with severe toxicity in retinoblastoma patients, including midface deformities, cataracts, retinopathy, optic neuropathy, and increased second tumour risk in patients with heritable disease.<sup>4,5</sup> To avoid these adverse effects, most specialists now utilise chemotherapy with focal therapy as a first line treatment for more advanced disease. Unfortunately, current chemotherapeutic regimens are also associated with significant morbidity in these paediatric patients, including infections, fever, gastrointestinal toxicity, neurotoxicity, and myelosuppression.<sup>6-8</sup> One of the aims of our research is to identify additional, less toxic agents with clinical utility in this disease.

Celecoxib (Celebrex, Pfizer, New York, USA) is a non-steroidal anti-inflammatory drug (NSAID) and anti-tumour agent that selectively inhibits cyclooxygenase-2 (COX-2).<sup>9</sup> COX family enzymes catalyse the rate limiting step in the synthesis of prostaglandins (PGs) from arachidonic acid. COX-1 is expressed constitutively in most tissues and mediates the synthesis of prostaglandins that control normal physiological functions. COX-2 is expressed in response to stimuli, including mitogens and cytokines, and mediates the synthesis of prostaglandins involved in inflammatory processes.

COX-2 was identified as a target for cancer therapy based on research in several areas (reviewed by Gasparini *et al*<sup>10</sup> and

Dannenber and Subbaramaiah<sup>11</sup>). Initial evidence arose from epidemiological studies showing that chronic use of NSAIDs (COX family inhibitors) reduces risk for several cancers. Subsequent research revealed that COX-2 is frequently overexpressed in many malignancies, and that COX-2 derived prostaglandins promote tumour growth by stimulating angiogenesis; by suppressing antitumour immunity; and by increasing tumour cell proliferation, invasiveness, and resistance to apoptosis. Findings of reduced tumour growth in COX-2<sup>-/-</sup> knockout mice and increased tumorigenesis in transgenic COX-2 overexpressing mice provided additional evidence of the importance of COX-2 in oncogenesis.

Consistent with these data, selective COX-2 inhibition with celecoxib potently inhibits tumour growth in animal models of colon, breast, lung, and other cancers.<sup>9,12-14</sup> Celecoxib's antitumour effects have been linked to an antiangiogenic action in several animal model systems,<sup>15-17</sup> and corneal angiogenesis assays confirm that this agent is strongly angiostatic.<sup>16,18</sup> Celecoxib appears to reduce neovascularisation by inhibiting production of angiogenic growth factors, including the COX-2 dependent prostaglandin E2 and vascular endothelial growth factor (VEGF).<sup>18,19</sup> Celecoxib also induces apoptosis in a variety of malignant cell types by mechanisms that appear to be independent of COX-2.<sup>16,20,21</sup> However, significant apoptotic effects are observed only at high, non-physiological concentrations (>20  $\mu$ M), suggesting that this mechanism is not relevant to the antitumour effects of celecoxib in vivo.

Human clinical trials of celecoxib have been initiated in patients with colorectal, breast, lung, and other cancers, and early results of these trials are promising.<sup>10</sup> For example, in a phase II trial, celecoxib enhanced the response of preoperative carboplatin and paclitaxel in patients with non-small cell

**Abbreviations:** COX-2, cyclooxygenase-2; EBRT, external beam radiotherapy; LH $\beta$ , luteinising hormone  $\beta$  subunit; NSAID, non-steroidal anti-inflammatory drug; PCR, polymerase chain reaction; PGs, prostaglandins; VEGF, vascular endothelial growth factor

lung cancer.<sup>22</sup> Celecoxib also significantly reduced the number of colorectal polyps in patients with familial adenomatous polyposis, a cancer predisposition syndrome associated with a nearly 100% risk for colon cancer.<sup>23, 24</sup>

COX-2 is overexpressed in 96% (28/29) of retinoblastoma tumours, suggesting that COX-2 is also important in retinoblastoma development.<sup>25</sup> To investigate the potential utility of celecoxib in the treatment of this disease, we tested the effects of this agent *in vitro* in retinoblastoma cell lines and *in vivo* in transgenic murine retinoblastoma.

## MATERIALS AND METHODS

### In vitro studies

#### Cell lines

Y79 and Weri-RB1 established human retinoblastoma cell lines were obtained from the UCSF Cell Culture Facility. Cells were maintained in a 5% CO<sub>2</sub> humidified atmosphere at 37°C in RPMI-1640 medium, supplemented with 15% fetal bovine serum, penicillin, and streptomycin.

#### Purification of celecoxib for in vitro studies

Celecoxib preparations (400 mg Celebrex capsules, Pfizer) were first washed with water at room temperature. After filtration, the residue was dissolved in methanol at 60°C. The filtrate was then evaporated to dryness and re-crystallised twice with methanol. Purity of the crystal was determined to be >99% by high performance liquid chromatography analysis under ultraviolet detection at 210 nm (flow rate 1 ml/min; column C-18, 4.6×250 mm, 5 µm particle size; mobile phase 50% CH<sub>3</sub>CN-0.1% H<sub>3</sub>PO<sub>4</sub>; retention time of celecoxib, 9.6 minutes). After purification, a 200 mM stock solution of celecoxib was prepared in DMSO and stored at -20°C.

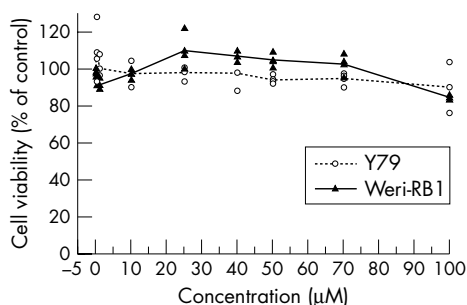
#### Antiproliferation assays

Y79 or Weri-RB1 cells were seeded into 96 well microtitre plates (10 000 cells/well) and treated with 0.1–100 µM of celecoxib or with vehicle control. At 96 hours post-treatment, live cell counts were determined by WST-1 cell proliferation assay (Roche, Penzberg, Germany), as per manufacturer's instructions. Viability results were expressed as a percentage of mean control values. All experiments were performed in triplicate.

### In vivo studies

#### Animals

The mice used in this study carry a transgene composed of the coding region of the simian virus 40 large T antigen (*TAg*) oncoprotein driven by the promoter of the human luteinising hormone β subunit (*LHβ*). At around 8 weeks of age, retinal *TAg* expression triggers the development of bilateral, multi-



**Figure 1** Antiproliferative effects of celecoxib in retinoblastoma cell lines. For each cell line, results of three replicate experiments are shown. Scatter points represent mean results of each replicate experiment (in which n=8), and lines represent the mean of all three experiments.

focal retinal tumours analogous to human retinoblastoma.<sup>26</sup> Heterozygous *TAg* positive mice were bred by mating heterozygous *TAg* positive males with normal CB6F1/J females (Jackson Laboratories, Bar Harbor, ME, USA). Pups were screened for the presence of the transgene by polymerase chain reaction (PCR) analysis, as previously described.<sup>27</sup> All experiments were performed with approval from the UCSF Committee on Animal Research.

#### Celecoxib preparation for in vivo studies

Celecoxib preparations (200 mg Celebrex capsules, Pfizer) were purchased from the UCSF inpatient pharmacy. Capsule contents were dissolved in DMSO at a concentration of 400 mg/ml celecoxib. Celecoxib in DMSO was then combined with sesame oil (14% v/v) and vortexed vigorously to create a suspension for oral administration.

#### Determination of maximum tolerated dose

Four groups of four transgene negative mice were treated once daily (M-F) for 2 weeks with 100 mg/kg, 150 mg/kg, 200 mg/kg, or 250 mg/kg celecoxib by oral gavage using a 20 gauge gastric feeding tube. Mice were monitored daily (M-F) for evidence of toxicity, including changes in appearance, behaviour, or body weight. Maximum acceptable toxicity was defined as weight loss ≤15%.

#### Treatments

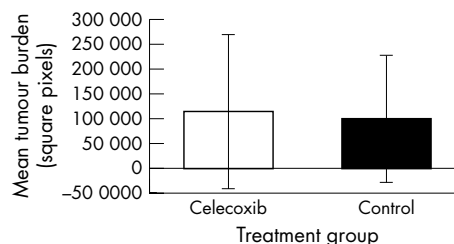
Two randomised groups of 24 transgene positive, 8 week old mice were treated once daily (M-F) for 6 weeks with 250 mg/kg celecoxib or with vehicle control by oral gavage. Mice were weighed twice weekly during treatment to monitor for toxicity.

#### Tissue collection and processing

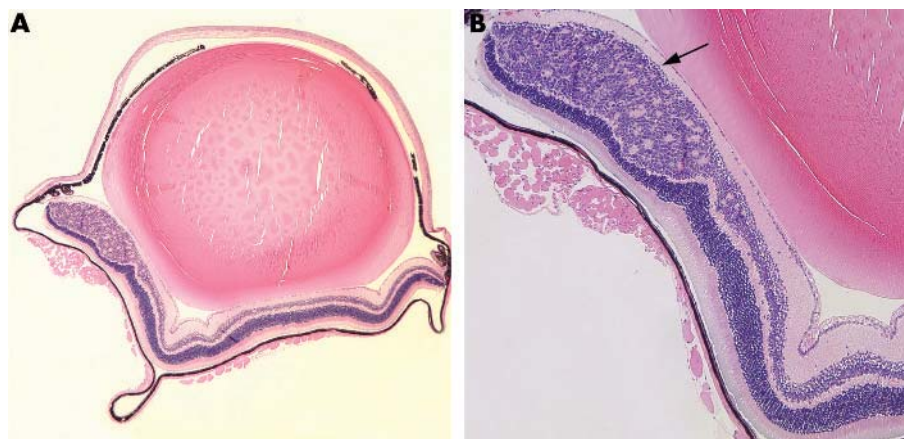
Mice were sacrificed on day 43 (at 14 weeks of age). Eyes were enucleated, formalin fixed, paraffin embedded, and serially sectioned. Sections were obtained from five levels throughout each eye and stained with haematoxylin and eosin. At time of sacrifice, tail specimens were obtained and frozen for future PCR testing to confirm transgene status in the event that any mice demonstrated no retinal tumour burden on histopathological analysis.

#### Determination of ocular tumour burden

Histopathological analysis of tissue specimens was performed in a masked fashion. Tumour foci were identified by light microscopy and digitally imaged at 100× magnification. The area of each tumour focus was then measured in square pixels using Scion Image software (Scion Corp, Frederick, MD, USA). The main outcome measure was mean tumour burden per level per mouse in each group.



**Figure 2** Ocular tumour burden in *LHβ-TAg* transgenic mice treated with celecoxib.



**Figure 3** Representative section depicting mean ocular tumour burden in treatment and control groups. Tumour area in this section is 109 658 square pixels (B, arrow), which is similar to mean tumour burden per level in treatment and control groups (114 560 and 99 793 square pixels, respectively (table 1)). (Stain, haematoxylin and eosin; original magnification  $\times 40$  (A) and  $\times 100$  (B).)

### Statistical analysis

The *t* test was used to test for differences in mean tumour burden between treatment and control groups. Results were expressed as mean values (SD). Significance was set at 0.05.

## RESULTS

### In vitro studies

Results of antiproliferation assays are shown in figure 1. These data show that celecoxib was ineffective at inhibiting proliferation of Y79 and Weri-RB1 cell lines, even at concentrations far exceeding clinically observed plasma levels ( $4.46 \mu\text{M}^{28}$ ).

### In vivo studies

#### Toxicity study

The maximum tolerated daily dose of celecoxib was determined to be 250 mg/kg, and this dose was chosen for subsequent therapeutic studies. Mean body weight change in mice treated at this dose was  $-7.1\%$  (range  $-3.4\%$  to  $-12.5\%$ ), which fell within acceptable values. There were no remarkable changes in appearance or behaviour.

#### Therapeutic study

All mice survived the study. There was no significant evidence of systemic toxicity as determined by body weight loss. Mean body weight change was  $+1.9\%$  (range  $-1.6\%$  to  $+5.8\%$ ) and  $+2.5\%$  (range  $+0.3\%$  to  $+4.6\%$ ) in treatment and control groups, respectively.

There was no significant difference in tumour burden between the treatment and control groups. Mean tumour area per level per mouse was 114 560 (SD 156 340) square pixels ( $n = 22$ ) and 99 793 (128 596) square pixels ( $n = 23$ ) in celecoxib and control groups, respectively ( $p = 0.73$ , table 1 and fig 2). Mean ocular tumour burden in treatment and control groups is depicted in a representative section in figure 3.

Three mice (two celecoxib treated mice and one control mouse) demonstrated completely normal retina in both eyes on histopathological examination, suggesting that initial

results of PCR analysis for transgene status in these mice were false positives. Results of repeat PCR analysis in these three mice using preserved tail samples confirmed that these mice were transgene negative. These animals were therefore excluded from the study.

## DISCUSSION

In an effort to identify additional, less toxic agents for the treatment of retinoblastoma, we performed preclinical studies of the COX-2 inhibitor and antiangiogenic agent, celecoxib, in this disease. Celecoxib has demonstrated potent chemopreventive and tumouristatic effects in other cancers.<sup>9</sup> However, its effects in retinoblastoma have not yet been described. Since COX-2 is overexpressed in the great majority of retinoblastoma tumours,<sup>25</sup> and because this malignancy is unusually vessel dependent,<sup>29</sup> we reasoned that celecoxib might be effective in the treatment of this disease. As an agent with limited toxicity, celecoxib promised to be particularly useful as a chemopreventive agent in patients with heritable retinoblastoma, who are susceptible to new tumour formation throughout their early years.

In the first part of our study, we evaluated the anti-proliferative effects of celecoxib in Y79 and Weri-RB1 human retinoblastoma cell lines. These studies demonstrate that celecoxib is ineffective at controlling retinoblastoma cell growth in vitro, even at concentrations as high as  $100 \mu\text{M}$ . In contrast, celecoxib does induce growth inhibitory and apoptotic effects in other malignant cell types, including breast, lung, and colon cancers.<sup>16 20 21</sup> However, significant cytotoxicity is observed in these cell types only at high, non-physiological concentrations ( $>20 \mu\text{M}$ ), suggesting that these effects are not relevant to celecoxib's antitumour action in vivo. Despite these negative results in vitro, it remained possible that celecoxib would control tumour growth in transgenic murine retinoblastoma through suppression of angiogenesis and other antitumour effects associated with COX-2 inhibition in vivo.

In the second part of our study, we treated 24 LH $\beta$ -TAg transgenic mice once daily (M-F) for 6 weeks with 250 mg/kg

**Table 1** Ocular tumour burden in LH $\beta$ -TAg transgenic mice treated with celecoxib

Group	Treatment	Tumour burden per level per mouse (square pixels)		
		Mean	SD	n
1	Celecoxib	114 560	156 340	22
2	Vehicle control	99 793	128 596	23

celecoxib. This dose represents approximately 20 times the human dose used in previous phase II clinical trials (800 mg/day), assuming an average human body weight of 70 kg.<sup>22–24</sup> To ensure that we captured any chemopreventive effects of this agent, mice were treated beginning at 8 weeks of age, when tumours are nascent,<sup>26</sup> through to 14 weeks of age, when tumours are large. Despite the high dosage and the early and prolonged treatment, we observed no significant difference in ocular tumour burden between treated and control mice.

It seems unlikely that the negative effects of celecoxib in this model could be attributed to insufficient retinal penetration by this agent. As a hydrophobic molecule that is 99.7% bound to plasma proteins, celecoxib's ability to penetrate the central nervous system is indeed limited.<sup>30</sup> However, celecoxib is a potent COX-2 inhibitor with a 50% inhibitory concentration of only 3–6 nM (measured by inhibition of prostaglandin E2 production).<sup>31</sup> Intraretinal levels well beyond this concentration are achieved in normal rats receiving just 12–15 mg/kg celecoxib by intraperitoneal injection.<sup>32</sup> The LHβ-TAg mice treated in this study would presumably have sustained even higher intraretinal drug levels, not only because of the ~20-fold higher dose administered (250 mg/kg, orally) but also because retinoblastoma formation is associated with disruption of the blood-retinal barrier and increased intraocular penetration of systemically administered agents.<sup>33–35</sup> Despite these considerations, it remains possible that improved retinal delivery of celecoxib could yield positive therapeutic results in this model. This hypothesis could be tested through subconjunctival delivery of celecoxib, which results in several-fold more effective retinal penetration than systemic delivery.<sup>32</sup>

As for the present study, our findings indicate that oral celecoxib therapy is unlikely to be effective in the clinical management of intraocular retinoblastoma. Despite these negative results, we remain hopeful that through careful testing we will eventually succeed in identifying effective agents with less toxicity for treatment of patients with retinoblastoma. These paediatric patients have considerable side effects from current chemotherapeutic regimens, and better treatments are clearly required.

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Competing interests: none declared

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