Abstract. Cyclooxygenase-2 (COX-2) is associated with tumour promotion, inhibition of apoptosis, angiogenesis and metastasis. Celecoxib, a selective COX-2 inhibitor was investigated, in patients with clinically localized prostate cancer using immunohistochemistry. Patients and Methods: Patients with cT1-2 prostate cancer (n=45) were randomized to celecoxib 400mg b.d. or no treatment for four weeks prior to radical prostatectomy. Histological sections of preoperative biopsy and matched radical prostatectomy specimens were stained for markers of cell proliferation (MIB-1/Ki-67), microvessel density (CD-31 with Weidner scoring), COX-2, apoptosis (TUNEL analysis), angiogenic factors (VEGF and KDR) and HIF-1. Results: Celecoxib decreased tumour cell proliferation, microvessel density, angiogenesis and HIF-1 whilst enhancing apoptosis. These effects approached statistical significance in a multivariate model and the cell proliferation index approached statistical significance on univariate analysis. Conclusion: In this pilot study a 4 week regimen of celecoxib resulted in measurable biological effects in prostate cancer tissue. These findings warrant further investigation.

Prostate cancer is the most common non-dermatological cancer in men in Western countries (1) and novel strategies of management are urgently needed. Celecoxib is a selective cyclo-oxygenase-2 (COX-2) inhibitor (coxib) that appears to have an anti-cancer effect in numerous in vivo and in vitro models (2). COX-2 is intimately involved in the development and progression of a variety of malignancies, including prostate cancer (3-5). COX-2 has been shown to interact with angiogenic, apoptotic, proliferative, invasive, metastatic and other pathways involved in cancer evolution (2, 6, 7). Chronic inflammation and oxidative stress are key players in COX-2-dependent carcinogenesis. Coxibs, most notably celecoxib, also exert their anti-cancer effects via COX-2-independent mechanisms including interference with Akt (signal transduction), NF-κB (inflammatory mediator of tumorigenesis), and other mediators of cancer development and progression (6, 7). The end result of both COX-2-dependent and COX-2-independent actions is an inhibition of cancer at multiple stages. Presently, the superior anti-cancer and cardiovascular safety profile of celecoxib make it the coxib of choice for clinical trials (8, 9).

The purpose of this study was to investigate the biological effects of celecoxib in early prostate cancer. Patients were randomized to receive either 400 mg celecoxib twice daily b.d. or no drug for four weeks prior to radical prostatectomy (RP). The effects of the drug were investigated, using immunohistochemistry for markers of cell proliferation, angiogenesis and its surrogate of microvessel density (MVD), hypoxia and apoptosis. Further, COX-2 expression was measured in the samples in order to ascertain that there were no significant differences in expression between any of the samples used in this assessment. It should be stressed that administration of a COX-2 inhibitor itself would not be postulated to bring about a decrease in the levels of the enzyme protein itself, rather its effects would be mediated via inhibition of the enzyme activity and downstream sequelae. Based on the literature it was hypothesized that
Patients and Methods

Patient selection and trial design. The study was a single-blinded, randomized, controlled phase II pre-surgical trial of celecoxib versus no drug for 28 days prior to radical prostatectomy (RP) for patients with clinically localized (cT1-2 NO MO) prostate cancer. Twenty-eight days was chosen as this was deemed the maximum time it would be ethically possible to defer surgery from the time of decision to undergo RP. Although patients were aware of which arm they had been randomized to, all authors were blinded to allocation. Patients who had decided to undergo RP as their management option were eligible. Their respective diagnostic TRUS biopsies were assessed for the same biomarkers as their respective RP samples. Patients taking non-steroidal anti-inflammatory drugs (NSAIDs) or other coxibs were excluded, as were those with relevant past history of these medications. Patients on hormonal manipulation and 5-ARIs (androgen receptor inhibitors; e.g. finasteride) were also excluded, because of the possible induction of high-grade tumours in these patients, given the recent evidence from the Prostate Cancer Prevention Trial (10). Patients with significant cardiovascular risk factors (ischaemic heart disease, hypertension, hyperlipidaemia, diabetes, smoking and peripheral heart disease) were excluded, though the evidence for adverse cardiac events with short-term celecoxib is limited (9). For the drug treatment arm patients were given 400 mg b.d. orally administered celecoxib (Celebrex®, Pfizer Corporation). The control group were given no treatment. Assuming an equal probability of the drug affecting the biomarkers by 20% in either direction (two-sided test), for the study to have 80% power, 40 patients were required. This sample size calculation was the best estimate given that there is no available literature on the likely magnitude of any change in biomarker score for this study. However, during the course of randomization it became clear that some pre-operative samples had no cancer on further sectioning and so five further patients were recruited in one further 2:1 block of randomization, totalling 45 patients (30 celecoxib: 15 control).

The CONSORT statement for the study. Of the 62 patients originally recruited, there were 17 dropouts (six from changes in operation date/ withdrawal from the operating list; five due to patients changing their minds as a result of media attention regarding the drug treatment arm and five due to accidental unblinding of the authors), leaving 45 patients who completed the study and formed the basis of the data analysis. The CONSORT flowchart is illustrated in Figure 1.

Sample collection. For pre-treatment assessment of biomarkers, TRUS samples, in the form of paraffin-embedded material, were sectioned for immunohistochemistry and analysed for the same biomarkers as the RP samples. For the RP samples, 45 prostatectomy specimens were cut up and the slides with the largest areas of tumour (referred to as the ‘index’ tumour) were identified; the corresponding block was then sectioned further for subsequent immunohistochemical staining.

Immunohistochemistry methods. Sections were stained using haematoxylin and eosin (H&E) for identification of the index tumour area. Ki-67 was measured using MIB-1 mouse monoclonal antibody (DakoCytomation, Ely, UK), CD-31 using the JC-70 mouse monoclonal antibody (DakoCytomation), COX-2 using the COX-2 mouse monoclonal antibody (Autogen Bioclear, Calne, UK), VEGF using the V1 mouse monoclonal antibody (obtained and generated in-house from NDCLS, University of Oxford, UK), KDR using clone P34a mouse monoclonal antibody (as for VEG1 antibody) and HIF-1 using the ESEE 122F8 mouse monoclonal antibody (Becton Dickinson, Oxford, UK). Immunohistochemical staining was performed as per conventional protocols i.e. dewaxing and rehydration, antigen retrieval, blocking of endogenous peroxidase, antibody-antigen binding, colouring the interaction, counterstaining and mounting (using Aquamount© or DPX according to the technique used). Positive and negative controls (in which tissues known to express the antigen of interest were included and the primary antibody omitted, respectively) were used.

MIB-1, CD-331, VEGF, KDR, HIF-1, COX-2 staining. Tissue sections were processed in the normal manner followed by antigen retrieval using citrate buffer at pH 6.0 (pH 9.0 with tris/EDTA for VEGF) with pressure cooking for 3 min followed by phosphate buffer solution (PBS) washing; for HIF-1 this was performed overnight in a 75°C water bath at pH 8.0 using sodium EDTA. Endogenous peroxidase activity was blocked using 10% hydrogen peroxide activity was blocked using 10%
peroxide in water for 5 min. The primary antibody was added (dilution 1:50 for MIB-1, 1:5 for CD-31, VEGF 1:2, KDR 1:5, HIF-1 1:20 and COX-2 1:200) for 30 min – 4 h according to method, followed by a PBS wash. Amplification of the antigen-antibody reaction was carried out by addition of a secondary antibody ChemMate DAKO Envision HRP (DakoMation, Ely, UK). DAB substrate complex was made up and used according to manufacturer’s instructions (ChemMate DAB; DakoMation). For COX-2 staining the chromogenic reaction used avidin/biotin (Vector Blocking Kit; Vector Laboratories Ltd, Peterborough, UK) for 20 min, as described (11). The biotinylated secondary antibody (Vector Laboratories Ltd.) was applied for 30 min, followed by Vectastain Elite ABC reagent (according to manufacturer’s instructions). Slides were then counterstained and mounted as above.

Figure 2. Part 1: A: Section of cancerous prostate tissue showing strong cytoplasmic staining for KDR expression (as indicated by arrows). B: COX-2 staining of cancerous prostate cancer glands (as indicated by arrows). C: MIB-1 staining in nuclei within prostate cancer glands (as indicated by an arrow). D: CD31 positive staining of endothelial cells within a field of prostate cancer glands (as indicated by arrows). Part 2: A: TUNEL positivity (as indicated by highly fluorescent areas marked by arrows) showing apoptotic nuclear fragmentation in a field of prostate cancer glands as visualized by light microscopy with an excitation filter. B: Illustration of underlying tissue architecture of TUNEL-stained, DAPI counterstained tissue section of prostate cancer glands as visualized by light microscopy with an excitation filter. C: Section of prostate tissue showing cancerous prostate glands with positive staining for VEGF adjacent to a non-staining group of benign glands. D: Section of prostate tissue showing HIF-1 nuclear staining in prostate cancer glands (as indicated by arrows). Part 3: Mean immunohistochemistry scores obtained for diagnostic (TRUS) biopsies (i.e. baseline scores) and for radical prostatectomy samples. Solid black bars denote control (non-drug treated) patients and shaded bars denote celecoxib-treatment group.


**Immunohistochemical scoring.** The cancerous areas on all stained slides were marked using the H&E section. All slides were scored blind by the lead author and a randomly selected 20% of all slides were scored by the study pathologist; kappa statistics to measure inter-observer variability were calculated. The results for MIB-1 were based on the percentage of positive cells counted within malignant glands in a number of high power fields, generally counting 500 cells if possible. The CD-31 count was used using the Chalkley method and also the Weidner method scanning at low power (12). This was decided on due to the small sample size obtained for TRUS biopsies which contained small numbers of cancerous cells in some instances. In order to ensure robustness in this particular assessment both methods were used and a correlation coefficient of 0.79 was obtained (data not shown). This resulted in two scores for each slide for each of the diagnostic biopsies (pre-drug baseline samples) whereas for the RP samples, due to the larger areas of cancer seen in the sectioned tissue, the Weidner method alone was used. Scoring of TUNEL staining was identical to that for MIB-1. The corresponding H&E sections were used to accurately identify malignant areas on the TUNEL-lined section. Positive apoptotic cells included TUNEL-positive, darkly stained nuclei or nuclear fragments with a cytoplasmic halo, as described (13). Again due to the small sample size obtained with the TRUS biopsy it was not possible to perform TUNEL staining on those samples. VEGF and KDR were scored on a scale of 0 (none) to 3 (strong) using visual inspection of several field containing cancerous glands. HIF-1 staining resulted in only moderate intensity as the maximum (designated 2), slight staining (designated 1) or no staining at all (designated 0).

As the data for COX-2 staining are inconsistent based on the available literature, it was decided to score both benign and cancerous areas of the tissue sections. The results obtained refer to staining in the nucleus. COX-2 staining was assessed semi-quantitatively by assessment of the intensity of staining: 0= none, 1=weak, 2=moderate, 3=strong; and by assessing the percentage of cells staining at each intensity: 1=0-10%; 2=11-50%; 3=51-80%; 4=81-100%. The final score for COX-2 staining was calculated by multiplying the two scores obtained (intensity and percentage) giving a final score from 0-12.

**Statistical assessment of immunohistochemistry data.** Kappa statistics were used to investigate inter-observer agreement in immunohistochemical scoring. Staining results were analyzed using t-tests, both unpaired and paired, for parametric comparisons and Mann-Whitney U-tests for non-parametric analyses. A Pearson correlation coefficient was calculated for comparison of Chalkley and Weidner scoring methods for microvessel density.

**Results**

**Side-effects of celecoxib treatment.** For the celecoxib treatment arm of the study, four patients were withdrawn from the trial due to medical side-effects. These were: indigestion (one patient), rash (two patients), and feeling generally unwell (one patient). No patient withdrew as a result of cardiovascular side-effects, and no evidence was found of any adverse cardiac events in any patient either during the trial itself or during the follow-up period thus far (median 32 m, range 20-44 m).

**Clinical baseline parameter.** The statistical analyses comparing the means of the clinical baseline parameters between the celecoxib and control groups showed no significant differences. These were for Control and Celecoxib treated groups respectively: PSA: 8.06 (3.47) and 8.25 (4.62), pathological stage 2.07 (0.26) and 2.10 (0.31), Gleason sum 6.53 (0.74) and 6.33 (0.71). Results are the means obtained with the standard deviation in parenthesis using a 2 sample t-test.

**Immunohistochemical scoring.** Examples of immunohistochemical staining of prostate cancer tissue from RP samples are shown in Figure 2. Univariate analyses (unpaired t-tests or Mann-Whitney U-tests) were used to compare the RP scores for the celecoxib and control groups for all immunohistochemical stains and are shown in Table I. Kappa statistics were calculated for each immunohistochemical stain in order to check inter-observer agreement. These values were for MIB-1 0.94, almost perfect; Weidner 0.73; COX-2 0.88; TUNEL 1.0; VEGF 0.89, KDR 0.86, HIF-1 0.78; the textual gradings for these levels of agreement are based on established criteria (14). Although, apart from MIB-1, none of the stains reached conventional levels of statistical significance i.e. p<0.05, it is important to note that the mean scores for the celecoxib group were lower for MIB-1, Weidner, VEGF, KDR and HIF-1, and higher for TUNEL as shown in Figure 2.

Multivariate analyses using logistic regression and multiple analysis of variance (MANOVA) approached statistical significance for the drug and control groups: p=0.058 and p=0.073, respectively.

A comparison of the means of immunohistochemical scores for baseline values obtained for diagnostic (TRUS) biopsies (i.e. pre-treatment) for the control and celecoxib treated groups revealed no statistically significant differences between the two groups. Hence, p-values for comparisons between the celecoxib and control groups were MIB-1 0.61; Weidner (microvessel density) 0.69; VEGF 0.08; KDR 0.24; HIF-1 0.71. The data indicated no evidence for a real difference in means between the two groups for COX-2 staining in benign areas p=0.38 and 0.11 for cancerous areas in TRUS samples. For the RP samples COX-2 in adjacent benign tissue p=0.61 and cancerous areas p=0.19, MIB-1 p=0.056, Weidner p=0.53, TUNEL p=0.26, VEGF p=0.83, KDR p=0.16 and HIF-1 p=0.16, for control versus celecoxib treated patients using a two sample t-test, with a pooled variance at 95% confidence interval.
Discussion

In this blinded, randomized study, analyses at baseline were performed and there were no significant differences between the two groups for any clinical or immunohistochemical parameters indicating the two groups were balanced. In the operative RP samples levels of staining between the two groups showed that scores for MIB-1, CD-31, VEGF, KDR, and HIF-1 were all reduced whereas apoptosis was increased following celecoxib treatment. Considering the p-values for these univariate analyses, only the MIB-1 result approached conventional levels of statistical significance. However, given the study was blinded all the trends observed support the working hypothesis. MVD is the most subjective of the immunohistochemical measurements performed (reflected in it having the lowest inter-observer level of agreement) and it is difficult to draw a conclusion from this isolated result.

Overall, the results of the univariate analyses suggest that celecoxib reduced HIF-1 expression in index tumour areas and increased the levels of tumour cell apoptosis. The fact that MIB-1 was scored quantitatively would have increased statistical power so that any difference in scores (6.2% in the control group versus 2.6% in the celecoxib group) almost reached conventional levels of statistical significance (p=0.056). It is not possible to pick up <4% differences in staining for the other biomarkers which are scored in a categorical fashion. Hence, it may be a limitation of the scoring technique for the semi-quantitative measures, rather than an absence of real biological effect, that was the cause of the p-values not reaching statistical significance. Whether this would translate into a significant biological effect for celecoxib has not been confirmed by this study, possibly for the reasons of under-powering as discussed above. It may be that future trials with larger accruals and longer durations of therapy will resolve this more conclusively. Previous work has shown that the COX-2 inhibitor etoricoxib is effective in prostate cancer patients given intermittent androgen deprivation therapy, as shown by significant decreases in PSA levels (15). In addition, a phase II study using celecoxib in recurrent prostate cancer given either radiation or radical prostatectomy showed that the drug delays in disease progression (16).

The current pilot study suggests that celecoxib may have anti-cancer effects on cell proliferation, apoptosis, angiogenesis and hypoxia. An assessment of gene expression profiling using a microarray based approach was also performed on prostate cancer biopsy samples from patients involved in this clinical trial. In that part of the study it was possible to see evidence of a pro-apoptotic effect of celecoxib on prostate cancer tissue (manuscript submitted). These findings are also in agreement with an earlier study of ours which looked at the effects of celecoxib in a COX-2 expressing prostate cancer model (17) and is further supported by another earlier study (18). There is enough data to support the rationale for a larger study to confirm these findings and to elucidate whether celecoxib could be a future therapeutic option in prostate cancer in the neo-adjuvant or indeed in the chemopreventative setting.

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References


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