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ORIGINAL ARTICLE

# A phase I pharmacodynamic study of the effects of the cyclin-dependent kinase-inhibitor AZD5438 on cell cycle markers within the buccal mucosa, plucked scalp hairs and peripheral blood mononucleocytes of healthy male volunteers

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**Abstract**
*Purpose* AZD5438 is a novel, orally bioavailable, cyclin-dependent kinase (CDK) inhibitor demonstrating preclinical pharmacodynamic (PD) effects on CDK substrates and active growth inhibition of human tumour xenografts. Clinical pharmacokinetic (PK) data shows its plasma $t_{1/2}$ to be 1–3 h. The main purpose of the current study was to evaluate PD activity of single oral doses of AZD5438 in healthy volunteers. Twelve healthy male subjects received 10, 40 or 60 mg AZD5438 or placebo in a rotating placebo crossover study design. Rapidly proliferating normal tissues [buccal mucosa, peripheral blood mononucleocytes (PBMCs) and plucked scalp hair] were sampled predosing, 1.5 h ($t_{max}$), ±6 h post-dosing. The primary PD endpoint, phospho-retinoblastoma protein (pRb) levels in buccal biopsies (unit length labelling index) assessed by immunohistochemistry, was used as a biomarker of CDK activity.
*Results* Phospho-pRb levels were demonstrated to decrease in an epitope, dose- and time-dependent manner. Statistically significant reductions in the ratio phospho-pRb/total pRb were detected at 1.5 h post-dose compared to placebo for both 40 mg [S807–S811 epitope geometric least-squares mean (glsmean) ratio = 0.75, $P = 0.014$] and 60 mg AZD5438 (S807–S811 epitope glsmean ratio = 0.74, $P = 0.011$; T821 epitope glsmean ratio = 0.72, $P = 0.031$). No statistically significant differences were noted at 6 h post-dosing, indicating a close PK–PD relationship between AZD5438 and target inhibition. No effects attributable to AZD5438 were detectable on phospho-p27, p27, Ki67 in the buccal mucosa; or on phospho-pRb (S249–T252 epitope), phospho-p27 or Ki67 in the sheath cells of plucked scalp hair, raising issues about the appropriateness of different detection methods/tissues for use as PD biomarkers. In ex vivo stimulated PBMCs, statistically and near-statistically significant anti-proliferative effects, with the suggestion of a dose–response effect, were noted on the incorporation of [$^3$H]-thymidine (stimulated/non-stimulated) at 10, 40 and 60 mg, compared to placebo, at 1.5 h post-dosing (glsmean ratio = 0.65, $P = 0.019$; 0.70, $P = 0.056$; 0.51, $P = 0.001$, respectively).
*Conclusions* The modest PD effect, short plasma $t_{1/2}$ and close PK–PD relationship suggest that multiple daily dosing or sustained release formulations at higher

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doses will be necessary for AZD5438 to achieve sustained inhibition of CDK in human cancers.

**Keywords** Cell cycle · AZD5438 · CDK · Pharmacodynamic · Buccal mucosa

**Abbreviations**

| | |
|---|---|
| CPU | Clinical pharmacology unit |
| UK | United Kingdom |
| mg | Milligram |
| kg | Kilogram |
| ml | Millilitre |
| DLT | Dose-limiting toxicity |
| SMC | Safety monitoring committee |
| MWTD | Maximum well-tolerated dose |
| PBMC | Peripheral blood mononucleocyte |
| Cpm | Counts per minute |
| ANCOVA | Analysis of covariance |
| glsmean | Geometric least-squares mean |
| CI | 95% Confidence intervals |
| CV | Coefficient of variance |
| ULN | Upper limit of normal |
| $t_{1/2}$ | Half-life |

## Introduction

Aberrations in cyclins and their associated cyclin-dependent kinases (CDKs) are commonly associated with human cancers, and a number of different therapeutic strategies directed against these molecules are in clinical development [1, 5, 12]. AstraZeneca has recently developed the small-molecule CDK-inhibitor, AZD5438, for use in the clinic as an anti-tumour agent [16].

In order to progress from G1 to S phase within the cell cycle, CDK2 interacts with cyclin E, which leads to phosphorylation of retinoblastoma protein (pRb) [6–8, 14]. AZD5438 is a potent inhibitor in vitro of the cyclin E/CDK2 complex ($IC_{50}$ 0.006 μM), the cyclin B1/CDK1 complex ($IC_{50}$ 0.016 μM) and the cyclin A/CDK2 complex ($IC_{50}$ 0.045 μM) (K. Byth et al., manuscript in preparation). Consequently, it was hypothesised that inhibition of CDK2 by AZD5438 would result in reduced phospho-pRb levels within affected cells. Preclinical in vivo experiments on AZD5438 (R. Wilkinson et al., manuscript in preparation) have shown activity in a wide range of human tumour xenografts, with tumour volume effects closely associated with inhibition of phosphorylation of pRb in the xenograft tissue (R. Wilkinson et al., manuscript in preparation). A first-in-man tolerability and pharmacokinetic (PK) study involving single ascending doses of AZD5438 conducted in healthy male volunteers demonstrated that the predominant dose-limiting toxicities of AZD5438 were nausea and vomiting in the absence of prophylactic supportive care [4]. The maximum well-tolerated dose (MWTD) was 60 mg, with $t_{max}$ occurring 0.5–3.0 h post-dosing and a plasma $t_{1/2}$ of 1–3 h. Utilising this information to determine both the doses to deliver and the timings of pharmacodynamic (PD) assessments, a normal tissue biomarker approach was employed in a further healthy volunteer study to provide PD proof of target modulation, evidence of anti-proliferative activity and an assessment of the PK–PD relationship of AZD5438 in man.

## Subjects and methods

### Study overview and healthy volunteer recruitment

A randomised, placebo-controlled, double blind healthy male volunteer study was conducted in the United Kingdom (UK) in full accordance with the Declaration of Helsinki and the International Committee on Harmonisation's guidelines on Good Clinical Practice. All study protocols, amendments and informed consent forms were approved in writing by an independent ethics committee.

Study specific inclusion criteria included scalp hair ≥5 mm in length, a body mass index between 18 and 30 kg/m$^2$, normal medical history, physical examination, routine blood tests and resting 12 lead ECG, as well as negative screens for serum Hepatitis B sAg, Anti-Hepatitis C antibody and anti-HIV antibody. Specific exclusion criteria included the use of any prescribed, non-prescribed or herbal medications (with the exception of paracetamol within the recommended daily dosage) within 3 weeks of the first dose of study drug, or receipt of another new chemical entity during the preceding 4 months. In addition, the use of tobacco-based products within 1 year of the first dose of study drug or the presence of any clinically significant oral disease was prohibited. Study specific restrictions included abstinence from consuming grapefruit, liquorice, cruciferous vegetables, alcohol or high caffeine-containing foods/drinks for 24–72 h before and after each dose of study drug, and abstinence from any concomitant medication or therapy (except paracetamol), unless deemed necessary by the study physician.

### Drug supply and administration

AZD5438 was supplied by AstraZeneca Investigational Products (Macclesfield, UK) in 2.5 and 20 mg tablets, with matching placebos. AZD5438/placebo

tablet(s) were administered orally in the semi-recumbent position with 240 ml of still mineral water.

## Pharmacodynamic study design

A four-period crossover study design with 14-day washouts was employed, using 12 volunteers (Fig. 1). The sample size, allowing for drop-outs, was based on the calculation that ten subjects would provide >90% power to detect a 40% reduction in the primary outcome variable (phospho-pRb), significant at the two-sided 5% level, given a 29% coefficient of variation in buccal biopsy tissue derived from a previous healthy volunteer methodology study [2]. Volunteers were given single oral doses (AZD5438 at 60, 40 and 10 mg or matching placebo) using a Latin-square design to allocate a dosing sequence to each across the four visits. The AZD5438 doses were based on the MWTD, two-thirds and one-sixth of the MWTD, respectively, derived from a previous first-in-man single ascending dose study [4]. Buccal biopsies (3 mm) and hair pluckings (30 usable hairs per time-point) were performed before each dose, at 1.5 and 6 h post-dosing with AZD5438/placebo. Blood samples for preparation of peripheral blood mononucleocytes (PBMCs) were taken pre-dose and at 1.5 h post-dosing. Based on the detailed PK information derived from the previous first-in-man study, a minimal number of PK time-points were analysed within this study with the aim of ensuring concordance with the previous data (pre-dose, 1.5, 6 and 24-h post-dose). Any adverse events reported by the subjects were graded according to the National Cancer Institute Common Terminology Criteria (CTC) Version 3, March 2003.

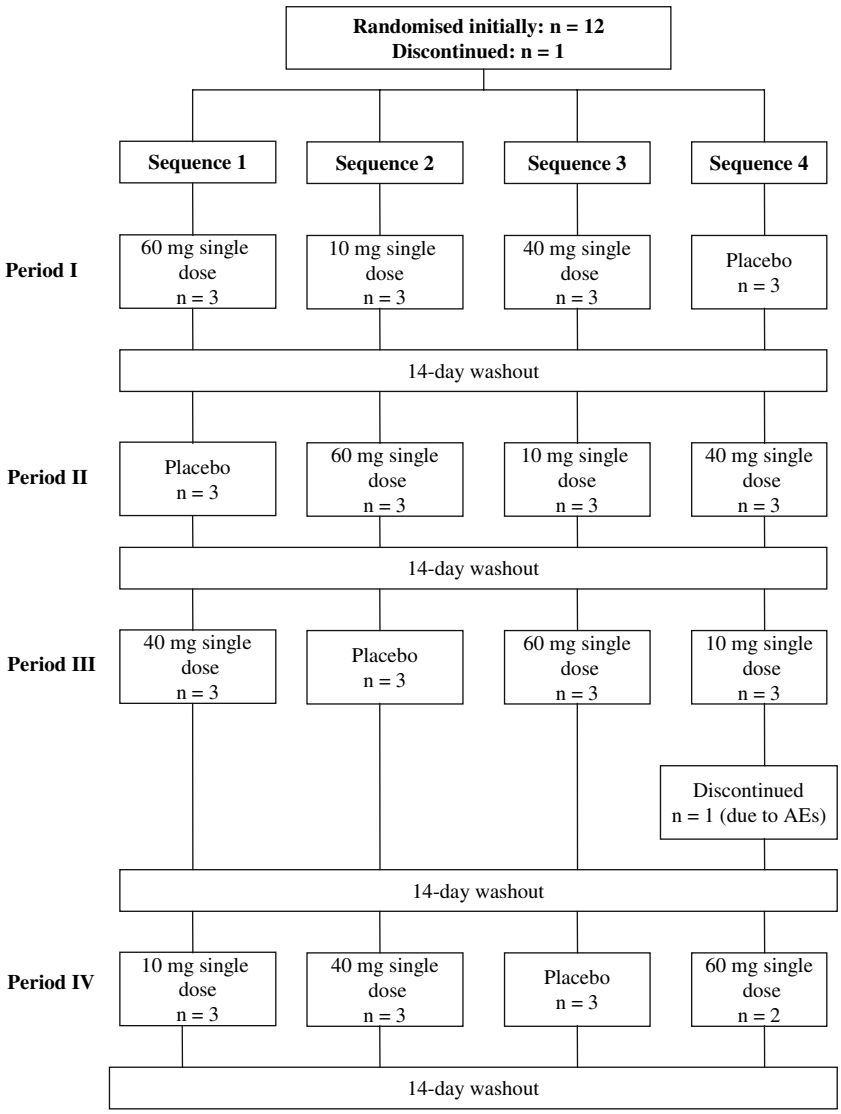


**Fig. 1** Flow chart showing volunteer disposition (completion or discontinuation) during study. In all cases, *n* refers to the number of subjects

AEs Adverse events

Supportive interventions to ameliorate toxicities were permitted, but prophylactic measures were not.

## Pharmacodynamic assessments

Single 3-mm buccal punch biopsies, composed of epithelium and the underlying connective tissue, were taken at each time-point along the occlusal line of the buccal mucosa, prepared and analysed as previously described [2]. Briefly, biopsies were fixed in neutral-buffered formalin for 48 h, embedded in paraffin wax, sectioned at 4 μm thickness and prepared for immunohistochemistry (IHC) to detect a range of different biomarkers. Image analysis and quantification was performed using the Zeiss KS400 system (Imaging Associates, Bicester, Oxfordshire, UK) using a bespoke macro programme linked to a Leica DMRB microscope. The number of positive nuclei was expressed per millimetre of basement membrane as a unit length labelling index (ULLI) [11].

Blood for PBMCs was drawn at each time-point into 7 ml × 7.5 ml lithium heparin monovette tubes. PBMCs and autologous plasma were separated immediately using buoyant density centrifugation (Ficoll paque plus, Amersham Biosciences Corp., Piscataway, NJ). PBMCs were resuspended in RPMI medium containing 0.5% human serum (Sigma, St Louis, MO, USA) and stored at 37°C until use. PBMC proliferation was evaluated through ex vivo [$^3$H]-thymidine uptake into the DNA of actively proliferating cells, measured in counts per minute (cpm). PBMCs were seeded in 96-well plates at $2 \times 10^5$ cells per well in 90% autologous plasma, 10% RPMI, 0.5% human serum. Ten replicate wells were seeded for stimulated and non-stimulated cells. Proliferation was stimulated by the addition of OKT3 anti-CD3 antibody to a final concentration of 10 ng/ml. Following incubation at 37°C (5% $CO_2$) for 42 h, cultures were pulsed with 1 μCi per well [$^3$H]-thymidine for a further 6 h. Cells were harvested onto filter mats and [$^3$H]-thymidine incorporation measured using a Wallac 1205 BetaPlate liquid scintillation counter (Perkin Elmer, Boston, MA, USA). Arithmetic means of the ten stimulated and ten non-stimulated wells were used for statistical analysis. Each stimulated sample was either analysed as the stimulated value per se compared to placebo readings or adjusted for the non-stimulated value by utilising the ratio of stimulated to non-stimulated cpms for each well.

Plucked scalp hairs were taken, prepared and analysed to produce nuclei positive per unit area information (nuclei/mm$^2$) and area fraction information (total number of positive pixels divided by the total pixel count) on a range of different biomarkers. The method used was as previously described [3], with the exception of using methanol fixation for 10–15 min as the initial step rather than acetone fixation. Ten usable hairs were analysed per marker. Briefly, following fixation, intact hairs were incubated with primary antibodies, prior to using the EnVision™ Plus HRP (horseradish peroxidase) detection system. The hairs were subsequently embedded in epoxy resin before tangential sectioning at 2 μm and image analysis. Data was averaged from three adjacent sections of each hair before statistical analysis.

The antibodies/techniques used for the IHC analysis of biomarkers within the buccal mucosa and plucked hairs are described in Table 1.

## Statistical analyses

The primary PD outcome variable of the level of phospho-pRb in buccal biopsy tissue at 1.5 and 6 h post-dose was used to investigate the effects of AZD5438 compared to placebo. Secondary outcome variables included the adjustment of the primary marker to account for the total Rb for each sample through the variable phospho-pRb/total pRb, levels of phospho-

**Table 1** Details of antibodies/techniques used for hair/buccal biopsy IHC

| Target | Supplier | Host/type | Catalogue number | Dilution | Detection system |
|---|---|---|---|---|---|
| Total-pRb | CST | Mouse monoclonal | 9309 | 1:100 | ChemMate EnVision+ |
| Phospho(S249/T252)-pRb | Biosource | Rabbit polyclonal | 44–584 | 1:50 | ChemMate EnVision+ |
| Phospho(S780)-pRb | CST | Rabbit polyclonal | 9307 | 1:50 | ChemMate EnVision+ |
| Phospho(S795)-pRb | CST | Rabbit polyclonal | 9301 | 1:50 | ChemMate EnVision+ |
| Phospho(S807/S811)-pRb | CST | Rabbit polyclonal | 9308 | 1:200 | ChemMate EnVision+ |
| Phospho(T821)-pRb | Biosource | Rabbit polyclonal | 44–582-G | 1:50 | ChemMate EnVision+ |
| Total-p27 | RDI | Rabbit polyclonal | P27CabrX | 1:100 | ChemMate EnVision+ |
| Phospho(T187)-p27 | Upstate | Rabbit polyclonal | 06–996 | 1:250 | ChemMate EnVision+ |
| Ki67 | Dako | Mouse monoclonal | M7240 | 1:100 | ChemMate EnVision+ |

CST—New England Biolabs (UK) Ltd, Hitchin, Hertforshire, UK; Biosource—Nivelles, Belgium; Upstate—Dundee, UK; RDI—Concord, MA 01742-3049, USA; Dako—Ely, Cambridgeshire, UK

*phospho-Rb* phosphorylated pRb, *phospho-p27* phosphorylated p27

p27 and an assessment of the levels of total pRb, total p27 and the ratio and phospho-p27/total p27 in buccal biopsies. The use of the ratios enabled investigation of whether any effects of AZD5438 on the phospho-markers reflected an effect on phosphorylation per se or on overall protein expression. Levels of Ki67 in buccal mucosa and the level of incorporation of [$^3$H]-thymidine in stimulated and the ratio of stimulated to unstimulated PBMCs were also used as secondary outcomes to investigate anti-proliferative effects of AZD5438. Effects on biomarkers present within plucked hairs were explored as tertiary outcomes.

### Buccal mucosa data

To assess whether there was a drug effect on the buccal mucosa markers an analysis of covariance (ANCOVA) model was used with factors fitted for the effects of treatment, sequence, volunteer-within-sequence, period and pre-dose (baseline) marker levels. PD effects were expected to be multiplicative and markers were log-transformed prior to analysis. A previous methodology study [2] indicated that assumptions with regard to the data being log-normal were appropriate. Geometric least-squares mean (glsmean) and 95% confidence intervals (CIs) estimated from the ANCOVA model were calculated by dose. In order to preserve the alpha level for the comparisons, analyses of phospho-pRb at 1.5 and 6 h post-dose were conducted separately with pairwise comparisons using a closed hierarchical procedure from the highest dose down to assess which, if any, doses exhibited a response different from placebo (glsmean ratios). If the highest dose did not show evidence of a drug effect, the lower doses for that marker/method were not tested further in the statistical analysis. The results between epitopes were expected to correlate. Consistency of findings was assessed for evidence of effect rather than a formal adjustment for multiple comparisons. For an individual comparison, $P < 0.05$ was considered as evidence of effect.

### PBMC data

A similar ANCOVA model was applied to the PBMC data. Separate analyses were undertaken for stimulated samples and corrected for non-stimulated samples by way of the ratio of stimulated to non-stimulated.

### Plucked hair data

An ANCOVA model was fitted to the endpoint of number of positive nuclei/mm$^2$ with factors for the effects of treatment, sequence, volunteer within sequence, period and hair stage. A treatment by hair stage interaction was also assessed to see if there was evidence that any of the stages affected the response of the volunteer to treatment.

## Results

In total 12 volunteers with a mean age of 45.0 years (range 24–63) and mean height and weight of 176.9 cm (range 170–188) and 81.2 kg (range 72–94), respectively, commenced the study with 11 volunteers completing. One volunteer, having previously received 40 mg and placebo, was discontinued in period 3 following dosing with 10 mg AZD5438 due to toothache and genital candidiasis, neither of which was considered related to study treatment (Fig. 1).

Pharmacokinetic parameters appeared broadly in concordance with those found in the previous healthy volunteer study (D.R. Camidge et al. 2006, manuscript submitted). AZD5438 showed rapid elimination with $t_{1/2}$ ranging from 1.40 to 2.97 h independently of dose, and plasma concentrations below the limit of quantification in 29 of 35 samples taken 24 h post-dose.

The number of adverse events increased with dose. Of a total of 28 adverse events that were considered to be possibly drug-related, 27 were CTC grade 1. Gastrointestinal side-effects were most common, with 5/12 volunteers experiencing nausea at 40 mg and 7/11 volunteers and 2/11 volunteers experiencing nausea and vomiting, respectively, at 60 mg. There were no consistent haematological, biochemical or electrocardiographic changes attributable to AZD5438.

### Buccal biopsies

There are multiple sites on pRb known to be phosphorylated by CDKs. Antibodies directed against five different phospho-epitopes on phospho-pRb (S807–S811, S780, T821, S795 and S249–T252) were used for IHC on the buccal biopsy specimens. The formal statistical analysis of both the phospho-pRb and phospho/total-pRb ratio values at 1.5 h post-dose is presented in Table 2 and the glsmeans presented graphically (for phospho/total-pRb ratio values at 1.5 and 6 h post-dosing) within Fig. 2. Statistical evidence of activity was apparent at 1.5 h post-dosing for both 40 and 60 mg when using antibodies directed against the S807–S811 epitope of phospho-pRb. When results were expressed as a ratio of phospho/total-pRb the statistical significance of the S807–S811 effects increased and effects on the T821 epitope became statistically significant at the

**Table 2** Analysis of ULLI compared to placebo phospho-pRb staining levels and the ratio of phospho-pRb/total-pRb in buccal biopsy tissue at 1.5 h post-dosing with AZD5438

| Variable | Statistic | Placebo | AZD5438 dose | | |
|---|---|---|---|---|---|
| | | | 10 mg | 40 mg | 60 mg |
| Phospho-pRb (S807–S811) | glsmean ratio[a] | 1.00 | 1.04 | 0.79 | 0.85 |
| | 95% CI | NA | 0.88–1.22 | 0.68–0.94 | 0.72–1.00 |
| | *P* value | NA | 0.627 | 0.007 | 0.050 |
| Phospho-pRb (S807–S811)/total-pRb | glsmean ratio[a] | 1.00 | 0.93 | 0.75 | 0.74 |
| | 95% CI | NA | 0.75–1.16 | 0.60–0.94 | 0.59–0.93 |
| | *P* value | NA | 0.509 | 0.014 | 0.011 |
| Phospho-pRb (S780) | glsmean ratio[a] | 1.00 | 0.94 | 0.75 | 0.83 |
| | 95% CI | NA | 0.61–1.43 | 0.49–1.15 | 0.54–1.28 |
| | *P* value | NA | NT | NT | 0.381 |
| Phospho-pRb (S780)/total-pRb | glsmean ratio[a] | 1.00 | 0.83 | 0.70 | 0.72 |
| | 95% CI | NA | 0.57–1.22 | 0.48–1.03 | 0.49–1.06 |
| | *P* value | NA | 0.331 | 0.066 | 0.090 |
| Phospho-pRb (T821) | glsmean ratio[a] | 1.00 | 1.01 | 0.92 | 0.87 |
| | 95% CI | NA | 0.78–1.31 | 0.71–1.18 | 0.66–1.14 |
| | *P* value | NA | NT | NT | 0.303 |
| Phospho-pRb (T821)/total-pRb | glsmean ratio[a] | 1.00 | 0.92 | 0.88 | 0.72 |
| | 95% CI | NA | 0.70–1.23 | 0.66–1.17 | 0.53–0.97 |
| | *P* value | NA | NT | 0.374 | 0.031 |
| Phospho-pRb (S795) | glsmean ratio[a] | 1.00 | 0.96 | 0.95 | 0.93 |
| | 95% CI | NA | 0.71–1.30 | 0.71–1.28 | 0.69–1.27 |
| | *P* value | NA | NT | NT | 0.647 |
| Phospho-pRb (S795)/total-pRb | glsmean ratio[a] | 1.00 | 0.84 | 0.89 | 0.80 |
| | 95% CI | NA | 0.58–1.21 | 0.62–1.29 | 0.55–1.17 |
| | *P* value | NA | NT | NT | 0.240 |
| Phospho-pRb (S249–T252) | glsmean ratio[a] | 1.00 | 1.03 | 0.96 | 1.03 |
| | 95% CI | NA | 0.86–1.23 | 0.81–1.15 | 0.86–1.23 |
| | *P* value | NA | NT | NT | 0.742 |
| Phospho-pRb (S249–T252)/total-pRb | glsmean ratio[a] | 1.00 | 0.91 | 0.90 | 0.90 |
| | 95% CI | NA | 0.72–1.16 | 0.71–1.13 | 0.70–1.14 |
| | *P* value | NA | NT | NT | 0.356 |

A closed hierarchical testing procedure was used to contrast the doses of AZD5438 with placebo—therefore, if no difference was observed for the comparison of AZD5438 60 mg with placebo, no further comparisons between the lower doses of AZD5438 and placebo were made

*CI* confidence interval, *glsmean* geometric least-squares mean, *NA* not applicable, *NT* not tested

[a] Ratio of the glsmean of each dose/placebo (i.e. treatment effect)

60-mg dose level ($P = 0.031$). The S780 epitope followed a similar pattern but failed to reach statistical significance at the 60-mg dose level ($P = 0.09$). No phospho-pRb epitopes showed statistically significant evidence of reduction compared to placebo at 6 h post-dosing. The strongest correlation between the phospho-pRb epitopes was seen for S780 and S807/S811 ($r^2 = 0.51$, $P < 0.001$). Statistical analysis showed no significant differences between AZD5438 and placebo at 1.5 or 6 h post-dosing for signal levels of phospho-p27, total p27, phospho-p27/total p27 ratio or Ki67 in the buccal mucosa (data not shown).

## [$^3$H]-thymidine incorporation into PBMCs

The formal statistical analysis of the incorporation of [$^3$H]-thymidine for both stimulated values and ratios of stimulated to non-stimulated values at 1.5 h post-dose is presented in Table 3. Using only the stimulated data compared to placebo, statistically significant effects were noted at 1.5 h post-dosing with 60 mg AZD5438 (glsmean ratio = 0.65, $P = 0.017$). When data was expressed as the ratio of stimulated/non-stimulated cells, statistically and near-statistically significant reductions, with a suggestion of a dose–response effect compared to placebo, were noted at 1.5 h post-dosing with 10, 40 and 60 mg AZD5438 (glsmean ratio = 0.65, $P = 0.019$; 0.70, $P = 0.056$; 0.51, $P = 0.001$; respectively).

## Plucked hairs

Antibodies directed against phospho-pRb (S249–T252), Ki67 and phospho-p27 were used for IHC on the plucked hair specimens. It was planned to acquire

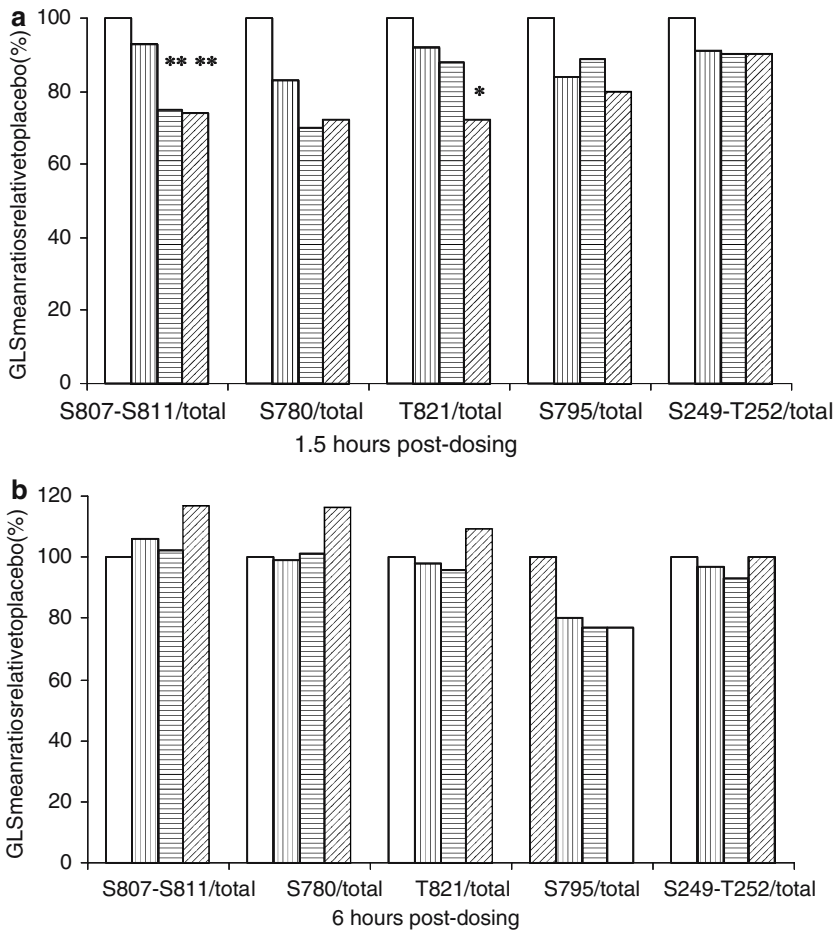


**Fig. 2** Buccal biopsies. Effect of AZD5438 10 mg (*square with vertical lines*), 40 mg (*square with horizontal lines*) and 60 mg (*square with slanting lines*), relative to placebo (*open square*) for AZD5438 on the ratio of phosphorylated-pRb/total pRb, in buccal biopsies at **a** 1.5 h and **b** 6 h after dosing. Each histogram represents the glsmean ratio relative to placebo, expressed as a percentage. Statistical significances are shown as $P < 0.05$ (*single asterisk*) and $P < 0.02$ (*double asterisks*), for comparison of AZD5438 dose versus placebo. All other values were not statistically significant ($P > 0.05$) relative to placebo

**Table 3** Analysis of cpm compared to placebo of [$^3$H]-thymidine uptake in stimulated PBMCs and analysis of the ratio of [$^3$H]-thymidine uptake in stimulated/non-stimulated PBMCs at 1.5 h post-dosing with AZD5438

| Time-point | Statistic | Placebo | AZD5438 dose | | |
|---|---|---|---|---|---|
| | | | 10 mg | 40 mg | 60 mg |
| 1.5 h | glsmean ratio[a] | 1.00 | 0.82 | 0.96 | 0.65 |
| | 95% CI | NA | 0.59–1.15 | 0.68–1.35 | 0.46–0.92 |
| | *P* value | NA | NT | 0.788 | 0.017 |
| 1.5 h | glsmean ratio[a] | 1.00 | 0.65 | 0.70 | 0.51 |
| | 95% CI | NA | 0.45–0.93 | 0.48–1.01 | 0.35–0.74 |
| | *P* value | NA | 0.019 | 0.056 | 0.001 |

A closed hierarchical testing procedure was used to contrast the doses of AZD5438 with placebo—therefore, if no difference was observed for the comparison of AZD5438 60 mg with placebo, no further comparisons between the lower doses of AZD5438 and placebo were made

*NA* not applicable, *NT* not tested

[a] Ratio of the glsmean of each dose/placebo (i.e. treatment effect)

30 hairs for each time-point, allocating 10 per marker. Hair wastage occurs at a number of steps along the processing pathway [3]. Expressed as a percentage of the maximum possible hairs, given the number of volunteers completing the study, quantitative IHC data were obtained for 80.4, 72.1 and 74.8% of hairs for phospho-pRb, Ki67 and phospho-p27, respectively. The remaining hairs were either rejected/lost during processing or there were problems with the sectioning. We had previously noted that plucked scalp hairs could be grouped morphologically into four different stages 0–3, based on the distance of the lower margin of the sheath from the base of the hair bulb [3]. Since there is some evidence that different sections along the sheath of plucked human hair may have different proliferative potentials [9], consideration of hair stage was considered important in interpreting any quantitative data on potential biomarkers of proliferation. The numbers of quantified hairs within each stage category were similar across all doses and time-points. For each marker a significant proportion of hair showed no nuclear staining at all. Of the 257 hairs analysed in stage 0, 246 had a zero result. Given that these hairs appeared anatomically different from the other stages, consistent with them being clubbed telogen hairs, it was felt that zero values in stage 0 hairs were more related to staging than drug effect and they were excluded from the final

analyses (7.4–9% of all hairs). Zero values in hairs from stages 1–3 could in theory represent either processing failures or genuine biological zeros. In order to determine whether they provided relevant data for incorporation into the analyses, the following logic was pursued: excluding hairs in stage 0, if the zero counts were genuine biological zeroes fewer such hairs would be expected to occur in those treated with placebo. In fact although the proportion of hairs with zero counts varied slightly between markers (12.3% phospho-pRb, 14.9% Ki67 and 23.1% phospho-p27), they were similar across treatment dose and, within dose, across time-points before and after dosing. On this basis the zero counts in all hairs were therefore assumed to be processing failures and were also excluded from the final analyses. Using the ANCOVA model, phospho-pRb, Ki67 and phospho-p27 were all noted to be higher in stage 1 hairs than in stage 3 hairs at 1.5 and 6 h post-dosing, in line with previous observations [3]. Although the raw data suggested a mean reduction of approximately 20% in the levels of phospho-pRb for 60 mg AZD5438 at 6 h post-dose compared to placebo, after adjusting for stage within the statistical analysis no treatment effect was discernible (data not shown). This discrepancy appeared to be due to a disproportionate amount of hairs in stage 3 assessed for phospho-pRb at the 6-h time-point for 60 mg AZD5438. There were no statistically significant treatment effects discernible within plucked hairs on the signal levels of either Ki67 or phospho-p27 (data not shown).

## Discussion

AZD5438 is a novel CDK inhibitor. It is orally bioavailable in man with a rapid $t_{max}$ and a relatively short plasma $t_{1/2}$ (D.R. Camidge et al. 2006, manuscript submitted). Side-effects from single doses up to 160 mg, in the absence of prophylactic supportive care, are predominantly gastrointestinal. Following the determination of PK and tolerability data on AZD5438 in healthy volunteers, this information was used to determine the doses employed and the timing of the assessments made within the current PD study. In order to establish proof of drug action on phospho-pRb, a target of CDK-inhibition, three separate normal tissue biomarker/non-tumour biomarker approaches were adopted: buccal biopsies, plucked scalp hairs and ex vivo stimulated PBMCs assessed at $t_{max}$ (1.5 h post-dosing) ± an additional assessment at 6 h post-dosing (in consideration of the potential for up to four times a day therapeutic dosing in the future). The study was sized according to the CV% of the primary PD variable, the ULLI of phospho-pRb within the buccal mucosa, determined within a previous healthy volunteer methodology study [2]. There are multiple phosphorylation sites on pRb, and antibodies directed against five different phospho-epitopes were employed (Table 1). The demonstration of a statistically significant reduction with two separate antibodies directed against phospho-pRb at 60 mg and with one antibody at 40 mg, along with a PD time course that parallels the PK of AZD5438 in the plasma strongly suggests that these effects on the target of CDK2 are AZD5438-related (Table 2; Fig. 2). The lack of effect at 6 h post-dosing with any of the anti-phospho-pRb antibodies, or of an effect detectable at 1.5 h with three of the five anti-phospho-pRb antibodies employed illustrates the importance of selecting the correct time-point and method in assessing PD biomarkers within studies that may be pivotal in deciding the extent or form of any subsequent development of a novel drug or formulation. Whether the lack of effect seen with certain of the anti-phospho-pRb antibodies reflects the different CVs of the different assessment methods (the CV from the methodology studies in hair and buccal mucosa were both derived from antibodies directed against S249–T252), their degree of dynamic response to CDK inhibition or simply their suitability/unsuitability for IHC is unclear.

One of the natural inhibitors of pRb within the cell cycle is p27, which is inactivated in part by phosphorylation (phospho-p27) via CDK2, leading to destruction within the proteasome [10, 13, 15]. No statistically significant changes attributable to AZD5438 were noted in the buccal mucosa in p27, phospho-p27, phospho-p27/total p27 or Ki67 (as a marker of proliferation). Although the background variability and dynamic range of these markers/methods may be responsible, the potential for the exposure to be too short to manifest significant downstream effects on some of these markers also has to be considered. The normal cell cycle takes around 24 h to complete in fast-dividing mammalian cells and with apparently complete onset and offset of AZD5438 action on phospho-pRb within 6 h after a single oral dose it would be surprising if major anti-proliferative effects, for example, on Ki67, were apparent. However, in the accelerated cell cycle produced in PBMCs stimulated ex vivo with anti-CD3 there was evidence of an anti-proliferative effect of AZD5438 at 1.5 h post-dosing with 60 mg compared to placebo (Table 3). Expressing results as a ratio of [$^3$H]-thymidine uptake in stimulated/unstimulated PBMCs also provided statistical evidence of a drug effect with 10 mg, with the 40-mg dose just failing to reach statistical significance (Table 3). This suggests that potentially

clinically relevant downstream consequences can occur following target inhibition by AZD5438.

No PD effects attributable to AZD5438 were detectable on phospho-pRb, Ki67 or phospho-p27 in plucked hair. Partly, this may reflect the larger CV of these biomarkers in hair compared to the buccal mucosa noted in previous methodology studies [3], as well as previously mentioned concerns about the specific antibodies used and the potential for downstream markers to be impacted upon by short drug exposures. In addition, because whole hairs were stained before sectioning, such that staining of different sections from the same hair for different biomarkers was not possible, we could not express phospho-pRb signals in plucked hairs as a ratio to total pRb, which seemed to provide a more robust statistical analysis within the buccal mucosa data. These results raise issues about the suitability/unsuitability of different normal tissues for biomarker approaches within early drug development studies.

Given the relatively short $t_{1/2}$ and close relationship between plasma drug levels and the PD effects of the drug in normal tissues, it is likely that AZD5438 will require multiple daily dosing via the oral route, or the development of a sustained release formulation. This assumes comparable timescales of PK and PD effects in malignant tissues/cancer patients and that clinically relevant downstream PD effects, for example on proliferation, would require sustained upstream target inhibition. The PD effects on phospho-pRb and stimulated proliferation in PBMCs at the MWTD of 60 mg were clearly discernible, establishing proof of AZD5438's activity in man, but were modest in extent (Tables 2, 3; Fig. 2). In preclinical models, the level of phospho-pRb inhibition with AZD5438 was closely associated with the degree of growth inhibition (R. Wilkinson et al., manuscript in preparation). Assuming that the target molecules within malignant tissue are no more sensitive to AZD5438 than normal tissue, and/or that tissue drug levels are comparable, it is likely that higher systemic exposures to AZD5438 and greater PD effects will be required to achieve clinical benefit in cancer patients. Although it is possible that tolerability to the acute gastrointestinal side-effects of AZD5438 may occur to some extent following repeated dosing, it is likely that the combination of higher and more prolonged dosing will require prophylactic antiemetic support for optimal tolerability within future efficacy studies.

The described approach of utilising a normal tissue biomarker based PD approach in healthy volunteers, quickly established proof of drug action on the relevant target in man, downstream anti-proliferative effects in a stimulated ex vivo system and early evidence of a dose–response effect. Taken together, these data strengthen confidence in AZD5438 as a potential anti-neoplastic agent in man. In addition, they should support a more rapid and appropriate dose and schedule determination of AZD5438 within future patient-based studies, reducing the chances of biologically inactive doses being administered to cancer patients during early phase drug development. Further clinical studies of AZD5438, utilising a number of different dosing regimens in cancer patients, are underway.

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