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Preclinical *in vitro* and *in vivo* evaluation of the potent and specific cyclin-dependent kinase 2 inhibitor NU6102 and a water soluble prodrug NU6301

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ABSTRACT

To facilitate the evaluation of CDK2 (cyclin-dependent kinase 2) as a cancer target, the *in vitro* and *in vivo* properties of NU6102 (O^6-cyclohexylmethyl-2-(4′-sulphamoylanilino) purine) and a water soluble prodrug (NU6301) were investigated. NU6102 selectively inhibited the growth of CDK2 WT (wild type) *versus* KO MEFs (knockout mouse embryo fibroblasts) (GI_{50} (concentration required to inhibit cell growth by 50%) 14 μM *versus* >30 μM), and was more growth-inhibitory in p53 mutant or null *versus* p53 WT cells ($p = 0.02$), and in Rb (retinoblastoma protein) WT SKUT-1B *versus* SKUT 1 Rb deficient cells ($p = 0.01$). In SKUT-1B cells NU6102 induced a G2 arrest, inhibition of Rb phosphorylation and cytotoxicity (LC_{50} 2.6 μM for a 24 h exposure). The prodrug NU6301 rapidly generated NU6102 *in vitro* in mouse plasma, and tumour NU6102 levels *in vivo* consistent with activity *in vitro*. Eight or 12 hourly dosing of 120 mg/kg NU6301 for 10 days was well tolerated in SKUT-1B tumour-bearing mice and inhibited Rb phosphorylation in tumour tissue. Two (8 hourly dosing) and 3 (12 hourly dosing) day tumour growth delay was observed ($p = 0.04$ and $p = 0.007$, respectively) following NU6301 administration. NU6102 and its prodrug NU6301 have pharmacological properties consistent with CDK2 inhibition, and represent useful tool molecules for the evaluation of CDK2 as a target in cancer.

1. Introduction

Unrestrained cellular proliferation is a central feature of malignancy, and aberrant cell cycle control has been demonstrated by studies of clinical tumour material and in laboratory models of cancer. Kinases are key regulators of the cell cycle, and numerous cell cycle kinase inhibitors have been developed and evaluated in clinical trials for the treatment of cancer.[1] Prominent amongst these agents are the cyclin-dependent kinase (CDK) inhibitors[1–3]; however, as with all small molecule ATP-competitive kinase inhibitors, the absolute specificity of CDK inhibitors and the question of which

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CDKs are the preferred targets for cancer therapy has not been resolved. Initially, CDK2 (cyclin-dependent kinase 2) was identified as a priority target, based on the effects of dominant negative (DN) CDK2 constructs on cell division[4,5] and the strong link between CDK2 and the molecular pathology of cancer (e.g. direct or indirect loss of endogenous peptide CDK inhibitors (p21/p27) and over-expression of CDK2 cyclin partners (cyclins E and A)). However, recognition that the effects of DN-CDK2 may be indirect (e.g. due to sequestration of cyclins), the failure of CDK2 siRNA or CDK2 antisense constructs to induce cell cycle arrest in a number of tumour cell lines,[6] and the viability of CDK2 knockout mice,[7,8] have brought into question the credential of CDK2 as a target in cancer and as well its role in the cell cycle.[9,10]

It is increasingly recognised that targeted anticancer agents will only be effective as cancer treatments when used in patients whose tumours have molecular defects or characteristics that predispose to sensitivity to the drug. In this respect, recent data suggest that CDK2 may still be a viable target in certain specific disease settings. Thus, it has been shown that CDK2 is involved in regulating Myc-induced senescence, using CDK2 –/– *versus* CDK2 +/+ MEFs and the CDK inhibitor CVT-313 in tumour cell lines,[11] and the CDK inhibitors purvalanol A and roscovitine are selectively toxic to Myc-transformed cells, but not to cells transformed by other oncogenes.[12] Although the latter authors attributed the effects of purvalanol A and roscovitine to CDK1 inhibition, the CDK selectivity of these compounds for human as opposed to starfish CDK1 is less pronounced (Lan-Zhen Wang, unpublished results). Similarly, CDK2 knockdown and roscovitine have been shown to be selectivity toxic to *MYCN*-amplified *versus* non-amplified neuroblastoma cells, a potential synthetic–lethal interaction,[13] and CDK2 has also been implicated in Ras-induced transformation.[14,15] Additional recent data that support the potential of CDK2 as a target for cancer treatment are the activity of a non-ATP competitive peptide-based inhibitor in a lung cancer xenograft model, and the effects of CDK2 siRNA and CVT-313 in B-cell lymphoma cell lines,[16,17] studies in melanoma,[18] as well as the possible involvement of CDK2 in DNA repair.[19,20] Taking these more recent data together with the limited CDK2-specifity of certain existing small molecule CDK inhibitors, there is a strong

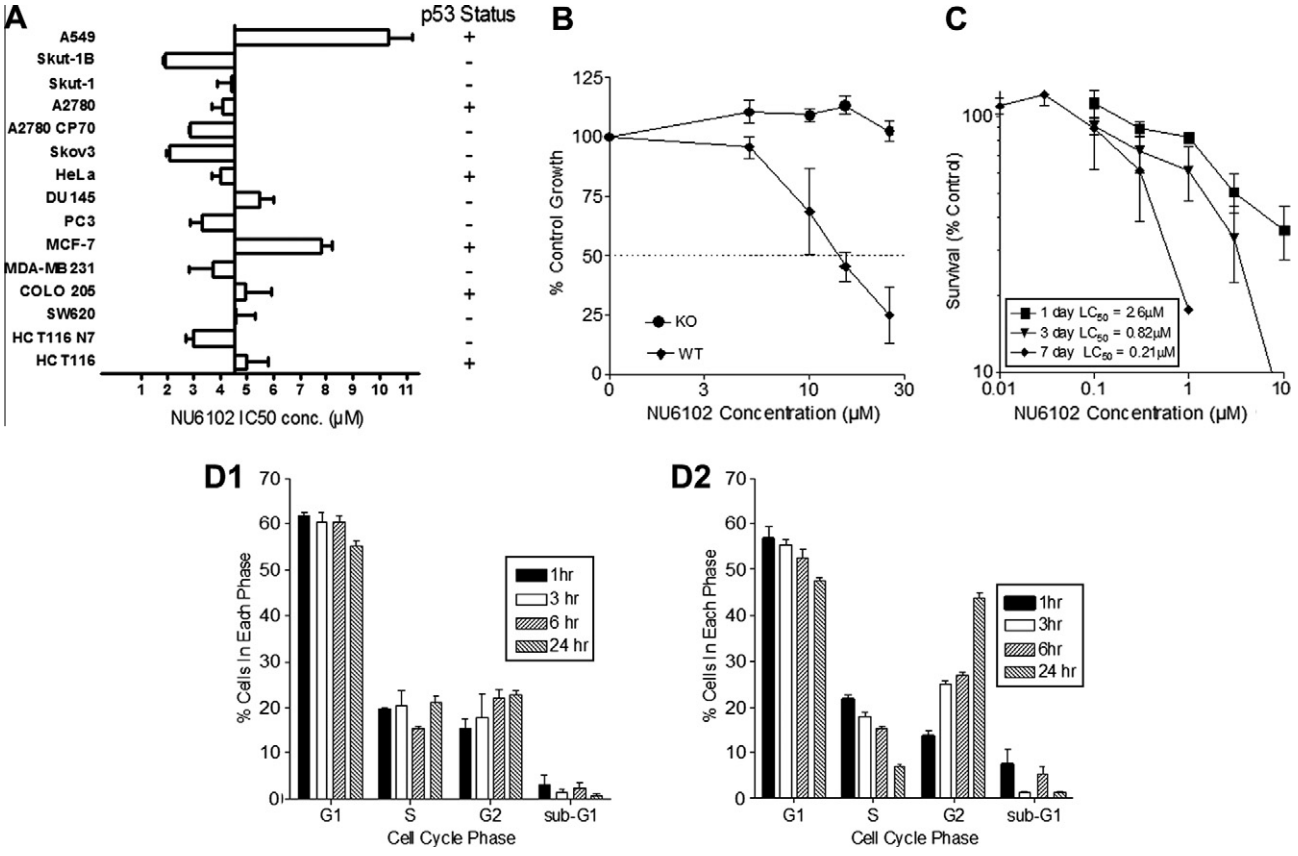

Fig. 1 – *In vitro* pharmacology of NU6102. (A) Sensitivity of a panel of cell lines to NU6102. GI_{50} and SD values for each cell line are shown relative to the mean GI_{50} value for all the cell lines (4.5 μM). (B) Growth inhibition of CDK2 WT (diamonds) and KO MEF (circles) as determined by SRB assay (data are mean and the bars the SD of 3 independent experiments). (C) Clonogenic survival of SKUT 1B cells demonstrating cytotoxic activity of NU6102 in the SKUT 1B cell line over 1–7 days (data are mean and the bars the SD of 3 independent experiments). (D) Effect of NU6102 on cell cycle phase distribution following 1 (solid bars), 3 (open bars), 6 (left hatched bars) and 24 (right hatched bars) hour exposure. NU6102 causes G2 arrest following treatment with 10 μM (Panel D2) compared to control (Panel D1) in SKUT 1B cells.

case for the further evaluation of potent and selective CDK2 inhibitors, and their use in chemical biology and target validation experiments.

We have previously described the design and discovery of O^6-cyclohexylmethyl-2-(4′-sulphamoylanilino)purine (NU6102, Fig. S6) using structure-based methods.[21] In isolated enzyme assays, NU6102 inhibits human CDK2 with a K_i of 6 nM and, although initial studies using starfish CDK1 suggested that NU6102 was equipotent again CDK1,[21] subsequent experiments demonstrated that NU6102 is at least 50-fold less active against human CDK1 and all other kinases tested.[22] NU6102 inhibits cell growth and causes cell cycle phase arrest in human breast cancer cell lines,[21,22] G2/M arrest in asynchronously growing cell lines and G1/S arrest in cells released from serum starvation,[22] and in *Xenopus* nuclei in a time-dependent manner.[23] Importantly, unlike many CDK inhibitors,[3] NU6102 is highly selective for CDK2 over CDK7 and CDK9 in enzyme assays (>200-fold) and does not inhibit RNA polymerase II phosphorylation at either serine 2 or serine 5 in cellular studies.[21]

NU6102 therefore represents a potentially useful probe molecule for the evaluation of CDK2 inhibition, and the current paper describes further *in vitro* studies to define the pharmacological properties of the molecule. Due to the poor physicochemical characteristics of NU6102, a water-soluble prodrug (N-acetyl-4-(6-cyclohexylmethoxy-9*H*-purin-2-ylamino)benzenesulphonamide potassium salt, NU6301, Fig. S6) was designed and developed for use in *in vivo* studies. Together, the data with NU6102 and NU6301 demonstrate that the molecules can inhibit cell proliferation and tumour growth, and these tool compounds are valuable for the further evaluation of CDK2 inhibition in defined experimental settings.

2. Materials and methods

2.1. Materials

General laboratory reagents were obtained from Sigma (Poole, UK) and HPLC grade solvents were purchased from Fisher (Loughborough, UK), unless otherwise stated. NU6102 was synthesised as previously described[21] and NU6301 as described in Supplementary Methods.

2.2. Methods

2.2.1. Cell culture

All cells, except CDK2 WT (wild type) and KO MEFs (knockout mouse embryo fibroblasts) which were a kind gift from Dr. S. Ortega, Dr. M. Malumbres, and Professor M Barbacid (Spanish National Cancer Centre, Madrid, Spain), were obtained from the European Collection of Animal Cell Cultures (Wiltshire, UK) and were grown in RPMI 1640 medium supplemented with 1000 units/ml penicillin, 100 μg/ml streptomycin (Gibco,

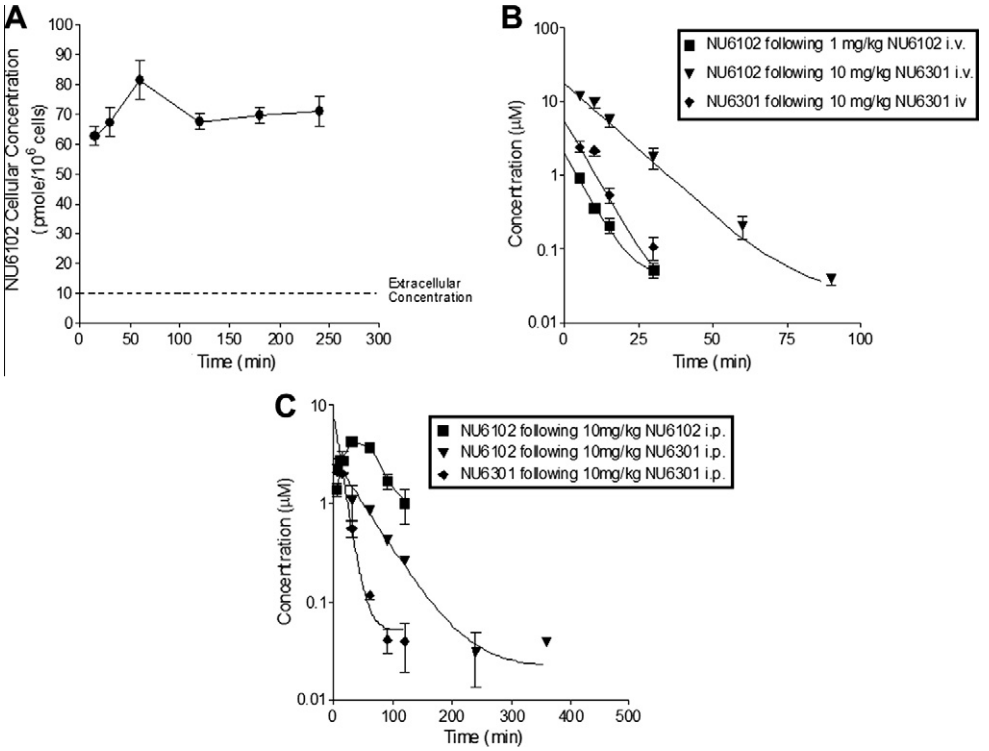

Fig. 2 – Cellular uptake and pharmacokinetics of NU6102. (A) Accumulation of NU6102 into SKUT 1B cells over time *in vitro* following exposure to an extracellular concentration of 10 μM (dashed line) NU6102 (data are mean and the bars the SD of 3 independent experiments). (B and C) Pharmacokinetics of NU6102 following i.v. (B) and i.p. (C) dosing of either NU6102 (squares) or NU6102 generated from the prodrug (inverted triangles). Note the maximum administrable dose of NU6102 was 1 mg/kg i.v. and 10 mg/kg i.p. The diamond symbols and lines show the concentration of the prodrug NU6301 (data are mean and the bars the SD of 3 animals *per* time point).

Paisley, UK) and 10% (v/v) foetal calf serum, unless otherwise stated. All the cells were maintained as exponentially growing cultures and were shown to be negative for mycoplasma contamination (MycoAlert, Lonza, Slough, UK).

2.2.2. *Growth inhibition and cytotoxicity assays*

The ability of NU6102 to inhibit cell growth was assessed in a panel of human cancer cell lines and CDK2 WT and KO MEFs using the SRB assay, as described in Supplementary Methods. Clonogenic survival of SKUT-1B cells, grown in MEM medium supplemented with non-essential amino acids (Gibco, Paisley, UK) and 10% (v/v) foetal calf serum, was measured as described in Supplementary Methods.

2.2.3. *Inhibition of CDK2-related phosphorylation of Rb and NPM*

The ability of NU6102 to inhibit phosphorylation of the Rb retinoblastoma protein at Threonine 821 (T821) and nucleophosmin (NPM) at Threonine 199 (T199) was assessed by western blot analysis as described in Supplementary Methods.

2.2.4. *Effects of NU6102 on cell cycle phase distribution in SKUT-1B cells*

Cell cycle analysis was carried out on SKUT1B cells exposed to 0, 3, 10, and 30 μM NU6102 for time periods of 1, 3, 6, and 24 h as described in Supplementary Methods.

2.2.5. *Cellular NU6102 uptake*

Semi-confluent cells were harvested and re-suspended in medium containing 10 μM NU6102, at 1×10^7 cells/ml, incubated at 37 °C in 5% CO_2 atmosphere and 150 μl (1.5×10^6 cells) aliquot was removed at time points for analysis of drug as described in Supplementary Methods.

2.2.6. *Measurement of drug concentrations*

Drug concentrations in plasma, tumour homogenates, media and cell suspensions were determined by HPLC as described in Supplementary Methods.

2.2.7. *In vivo studies*

All of the *in vivo* experiments were reviewed and approved by the institutional animal welfare committee, and performed according to National Cancer Research Institute Guidelines and national law. Detailed pharmacokinetic and pilot toxicity studies were performed in female Balb/C mice (Charles River, Ramsgate, Kent, UK). Tissue distribution, pharmacodynamic and efficacy studies were carried out in female athymic CD1 nude mice (Charles River, UK) implanted with SKUT-1B xenografts (1×10^7 cells in 50 μl of media injected subcutaneously into the right flank), maintained and handled in isolators under specific pathogen free conditions.

2.2.8. *Pharmacokinetic studies*

Plasma pharmacokinetics was determined following intravenous (i.v.) or intraperitoneal (i.p.) administration. Mice were treated with either the maximum administrable dose of NU6102 i.p. (10 mg/kg in a vehicle of 40% (v/v) polyethylene $glycol_{400}$) or i.v. (1 mg/kg i.v. in a vehicle of 10% (v/v) polyethylene $glycol_{400}$), or a dose of 10 mg/kg NU6301, equivalent to 8.4 mg/kg NU6102, in sterile saline for both i.v. and i.p. administration. Samples were collected and analysed as described in Supplementary Methods. Pharmacokinetic parameters were calculated using non-compartmental analysis and the terminal elimination rate estimated by log-linear regression.

2.2.9. *Determination of the maximum tolerated dose of NU6301*

The maximum tolerated dose of NU6301 given daily for 5 days to Balb-C mice was determined by administration of increasing doses of NU6301 i.p. Acute toxicity was assessed by measuring core body temperature with a rectal probe, and general toxicity over a two week period by observing the clinical condition of the animals and body weight measurement.

2.2.10. *Tumour pharmacokinetic and pharmacodynamic studies*

To determine if biologically active concentrations of NU6102 could be achieved in tumours following treatment with the MTD of NU6301, CD-1 nude mice bearing SKUT1B human tumour xenografts ($n = 3$/time point) were treated with NU6301 120 mg/kg i.p. Inhibition of T821 phospho-Rb was assessed in tumour as described in Supplementary Methods.

2.2.11. *Determination of in vivo antitumour activity*

Mice bearing SKUT-1B human tumour xenografts were treated with 120 mg/kg NU6301 every 8 h or every 12 h for 10 days. Tumour volume was monitored by calliper measurement using the equation $a^2 \times b/2$, where a is the smallest measurement and b the largest, and NU6102 and NU6301 concentrations were determined in plasma and tumour homogenates 1, 3 and 6 h after a single dose of 120 mg/kg NU6301. Data are presented as median relative tumour volumes (RTV), where the tumour volume on the initial day of treatment (day 0) is assigned an RTV value of 1.

3. Results

3.1. *NU6102 inhibits cell growth in a CDK2-, p53- and Rb-dependent manner*

The mean GI_{50} (concentration required to inhibit cell growth by 50%) for the panel of cell lines was 4.5 ± 2.2 μM, ranging from 1.9 ± 0.05 μM to 10 ± 1.6 μM (Fig. 1A). Although the range was limited, the mean NU6102 GI_{50} in the p53 mutant and null cell lines was significantly lower ($p = 0.021$ unpaired *t*-test) than in p53 WT cells. For two matched pairs of cell lines, p53 WT A2780 and HCT116 cells were less sensitive than A2780 CP70 p53 mutant cells (GI_{50} 4.1/2.9 μM, $p = 0.05$), and p53 degraded HCT116 N7 line (GI_{50} 5.0/3.0 μM, $p = 0.06$), although only at the 10% level in the latter case (Fig. 1A).

Consistent with CDK2 as the target for NU6102-induced cell growth inhibition the GI_{50} for NU6102 was lower in CDK2 WT MEFs than in CDK2 KO MEFs; 14 μM compared to >30 μM (Fig. 1B), although CDK2 KO MEFs were sensitive to NU6102 at higher concentrations (Fig. S8). In the paired Rb null SKUT 1 and Rb WT SKUT1B cells cell lines, SKUT-1B cells ($GI_{50} = 2.1 \pm 0.2$ μM) were significantly more sensitive than SKUT 1 cells (GI_{50} 4.4 ± 0.9 μM) ($p = 0.01$ unpaired *t*-test, *Fig.*

S1). NU6102 reduced SKUT-1B colony formation in a concentration and time-dependent manner, with LC_{50} values 2.6, 0.82, and 0.21 μM following 24 h, 72 h, and 168 h exposures, respectively (Fig. 1C).

In order to achieve a substantial reduction in phospho-T821 Rb or phospho-T199 NPM in SKUT-1B cells, NU6102 concentrations of ⩾10 μM were required (Fig. S2) and analysis of the effect of NU6102 on cell cycle phase distribution at 10 μM demonstrated a G2 arrest with a corresponding reduction in the percentage of cells in S phase (Fig. 1D).

The uptake of NU6102 was investigated in SKUT-1B cells where NU6102 was readily detectable at concentrations approximately 6–7-fold higher than in the culture medium (Fig. 2A). Uptake was also extensive and rapid in MCF-7 and A2780 cells (Fig. S3).

3.2. *NU6301 is an efficient prodrug of NU6102 that facilitates the administration of pharmacological doses of the CDK inhibitor*

Due to the poor solubility of NU6102, the N-acetyl potassium salt prodrug of NU6102 (NU6301) was developed and shown to be efficiently converted to the parent *in vitro* as described in the Supplementary Methods (Fig. S6). The pharmacokinetics of both the parent NU6102 and the prodrug (NU6301) were determined following i.v. and i.p. administration (Fig. 2B and C). The limited solubility of NU6102 meant the maximum administrable dose was 1 mg/kg i.v. and 10 mg/kg i.p., whereas NU6301 was administered at 10 mg/kg both i.v. and i.p. (equivalent to 8.4 mg/kg NU6102). NU6102 was liberated following either i.p. or i.v. administration of NU6301, and following i.v. administration peak plasma levels of 2.4 μM NU6301 and 12 μM NU6102 were observed 5 min post administration, whereas following administration of the maximum administrable dose of NU6102 i.v. the peak concentration achieved was 0.92 μM. The plasma half-life of NU6102 liberated following administration of NU6301 was 42 min following i.p. and 10 min following i.v. administration.

Plasma concentrations of ⩾3 μM NU6102, commensurate *in vitro* activity, were not achieved following i.p. administration of either NU6102 or NU6301 at 10 mg/kg, and only transiently following i.v. dosing of NU6301 at 10 mg/kg. A dose escalation study was performed to investigate the repeated daily administration of NU6301 at a higher dose. The dose was initially escalated to 120 mg/kg i.p. (equivalent to 100 mg/kg NU6102) with no major adverse effects. When the dose was further increased to 300 mg/kg (equivalent to 250 mg/kg NU6102) an 8 °C drop in body temperature and 9% decrease in body weight were observed after a single dose (Fig. 3A and B). Repeated daily dosing of 120 mg/kg NU6301 did not induce acute toxicity and had very little effect on body weight (Fig. 3B).

Tumour and plasma pharmacokinetics were studied following the administration of 120 mg/kg NU6301 at limited time points (1 h, 3 h, and 6 h). Rapid conversion of NU6301 to NU6102 was observed with prodrug only detectable at 60 min in plasma (24 ± 13 μM). Plasma concentrations of NU6102 were maximal at 60 min at 135 ± 30 μM declining to 0.7 ± 0.5 μM by 360 min. Tumour levels of NU6102 at 60 min were 77 ± 4 μM and were maintained at 180 (52 ± 24 μM) and 360 (15 ± 3 μM) min (Fig. 4 A) and after 5 days of dosing (Fig. S4). Having demonstrated that potentially active NU6102 levels could be achieved, an eight hourly dosing schedule was investigated in an attempt to maintain the concentration of NU6102 in tumour tissue. Eight hours after a single dose of 120 mg/kg NU6301, NU6102 was still present in tumour tissue at levels (9.3 ± 4.4 μM) consistent with those required for *in vitro* activity despite being at the limit of detection in plasma (0.14 ± 0.09 μM). Following subsequent doses the tumour level was 12 ± 8 μM 8 h after a second dose and 20 ± 3 μM eight h after the third dose (Fig. 4B), suggesting limited accumulation.

Western blot analysis of tumour disaggregates showed that T821 phosphorylation of Rb was inhibited for up to 6 hours following a single dose of NU6301 of 120 mg/kg (Fig. S5). Densitometry analysis of the western blot indicated that the percentage of total Rb phosphorylated at T821 was significantly reduced from 15 ± 1% in tumour samples from control (saline treated) mice to 8.1 ± 1.7 ($p = 0.004$), 11 ± 1.8 ($p = 0.034$) and 11 ± 1.8 ($p = 0.022$)% in tumours removed 1, 3, and 6 h, respectively, after treatment with NU6301 120 mg/kg i.p. (Fig. 4C).

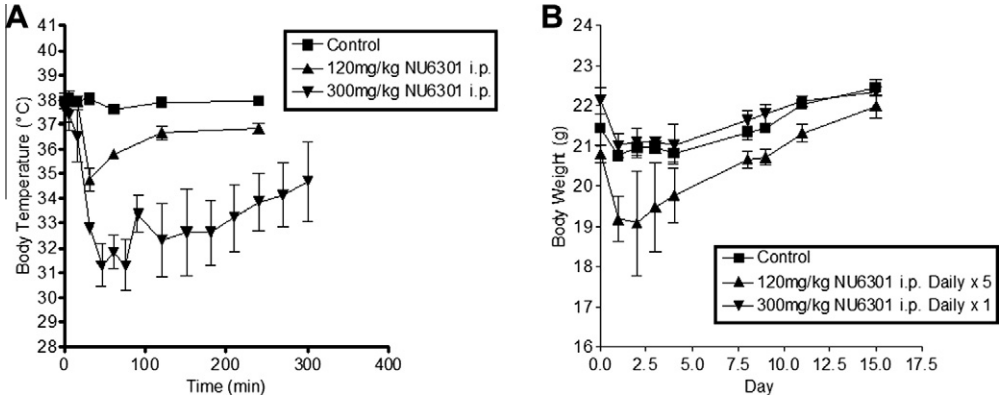

Fig. 3 – Toxicity of a daily × 5 schedule of NU6301. (A) Acute effects of NU6301 on body temperature (data are mean and the bars the SD of 3 animals *per* time point). Animals treated with 300 mg/kg NU6301 showed a severe drop in body temperature and dosing was not continued after day 1. Results for this group are for a single bolus dose (B) The chronic effects on body weight of increasing doses of NU6301 (data are mean and the bars the SD of 3 animals *per* time point). See note above.

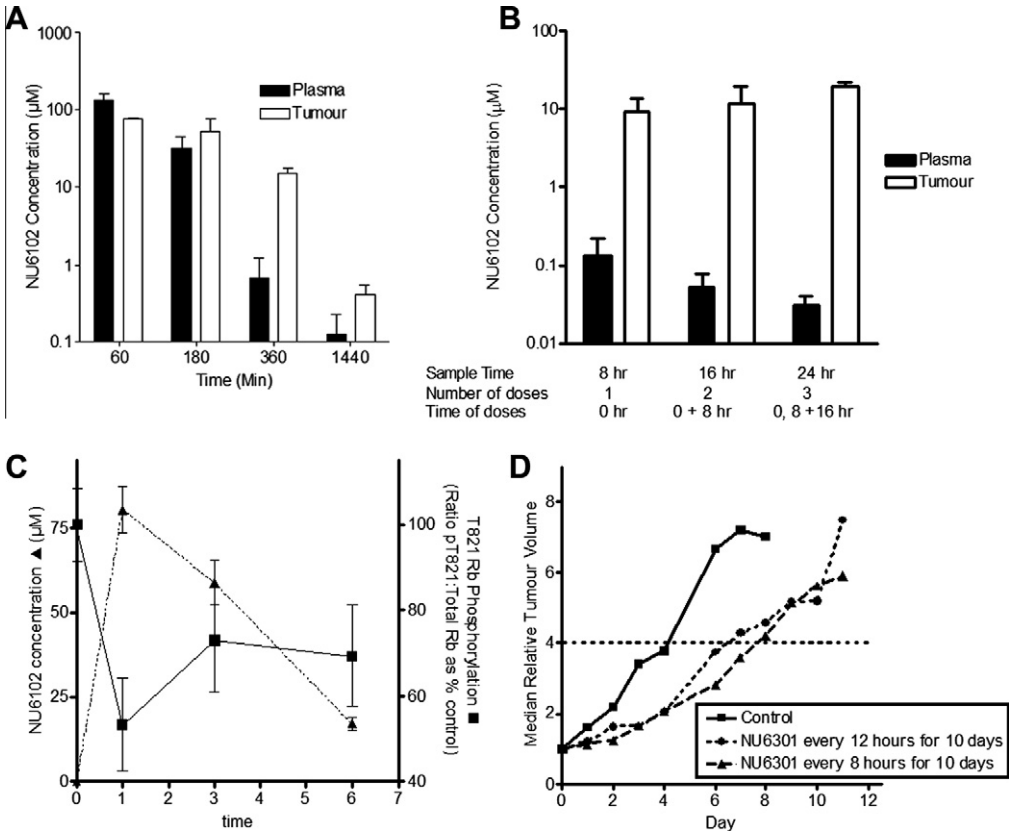

Fig. 4 – Pharmacokinetics, pharmacodynamics and efficacy of NU6301. (A) Plasma and tumour concentrations (μM) of NU6102 after a single 120 mg/kg i.p. dose of NU6301 (data are mean and the bars the SD of 3 animals *per* time point). (B) Plasma and tumour concentrations (μM) 8 hours after either a single or multiple (8 hourly) doses of 120 mg/kg NU6301 i.p. (data are mean and the bars the SD of 3 animals *per* time point). (C) Inhibition of T821Rb phorphorylation in SKUT1B tumour xenografts following treatment with a single dose of 120 mg/kg NU6301 (data are mean and the bars the SD of 3 animals *per* time point). (D) Growth of SKUT 1B xenografts treated with either twice (12 hourly) or thrice (8 hourly) daily NU6301 at 120 mg/kg i.p. for 10 days. No adverse effect was observed in the treated groups (data are mean and the bars the SD of 7 animals *per* time point, and the dashed line shows the point at which tumours reached four times the initial volume RTV 4).

3.3. *NU6301 has significant activity against the SKUT-1B human tumour xenograft following repeated administration*

Following treatment of mice bearing the SKUT-1B human tumour xenograft with 120 mg/kg NU6301 either twice or three times daily for 10 days no toxicity was observed as measured by body weight loss with nadirs of 97 ± 2% in control the group compared to 94 ± 6% and 96 ± 6% in the twice and three times daily treatment groups, respectively (Fig. S7). Repeated 8 hourly or 12 hourly dosing of NU6301 at 120 mg/kg for 10 days caused a significant (Mann–Whitney test) increase in the median time for tumour volume to quadruple, with a time to RTV 4 of 8 days ($p = 0.038$) for twice daily dosing, and 9 days ($p = 0.007$) for three times daily dosing, compared with 6 days for the control group (Fig. 4D).

4. Discussion

CDKs remain attractive targets for anti-proliferative cancer chemotherapy because of their central role in cell division and the frequent loss of endogenous peptide CDK inhibitors in malignancy, either directly (e.g. due to mutation, deletion or epigenetic silencing) or secondary to tumour suppressor gene loss (e.g. p53). A large number of CDK inhibitors have been or are still in clinical trials; however, with the exception of flavopiridol for the treatment of chronic lymphocytic leukaemia (CLL),[24] no major clinical antitumour activity has been reported to date. Furthermore, the activity seen with flavopiridol in CLL is ascribed to the inhibition of the transcriptional kinase CDK9, and consequent effects on the levels of anti-apoptotic proteins such as MCL-1. Thus cell cycle CDKs as cancer drug targets lack clinical validation.

Amongst the cell cycle CDKs, CDK2 has attracted the most attention and its exploitation as a drug target has been facilitated by the early availability of crystallographic data that in turn have allowed structure-based drug design. Using this approach a number of very potent CDK2 inhibitors have been developed; however, as with all ATP-competitive kinase inhibitors, the specificity of these compounds for CDK2 over other CDKs, and indeed other kinases, is always an issue. We have previously described the development of NU6102, using structure-based drug design, and aspects of the *in vitro* pharmacology of the compound.[21,22] In kinase inhibition studies NU6102 displays selectivity for CDK2; however, hitherto, definitive

evidence of CDK2 selectivity in a cellular model and demonstration of activity in *in vivo* models has been lacking.

In the current study, evidence for the selective activity of NU6102 against CDK2 in a cellular context was obtained by comparison of the activity of the compound in WT and CDK2 KO MEFs. Encouragingly, whilst NU6102 inhibited the growth of the WT MEFs at concentrations of <30 μM, no growth inhibition in the CDK2 KO MEFs was observed at concentrations ⩽30 μM. Given that CDK2 KO mice are viable,[7,8] and hence kinases other than CDK2 must be able to maintain cell division in the absence of the enzyme, the ability of NU6102 to inhibit the growth of the CDK2 WT MEFs appears to be inconsistent with the molecular genetic data, particularly as in a number of tumour cell lines depletion of CDK2 using siRNA also fails to inhibit cell division.[6] However, it is important to note that in both gene-knockout mice and following mRNA depletion, CDK2 protein will be absent. In contrast, in cells treated with a small molecule CDK2 inhibitor catalytically inactive protein will be present which has the potential to act in a dominant negative manner. This key difference between the effects of small molecule CDK2 inhibition and CDK2 protein depletion or ablation may explain the reason for the apparently discrepant results obtained with the two approaches.

The differential activity of NU6102 against p53 WT and non-functional cells, and against Rb WT and null cells, is also consistent with CDK2 as the primary cellular target for the compound. Thus, in the small cell line panel studied, p53 non-functional cell lines were in general more sensitive than p53 WT cells, a difference that is potentially due to the loss of endogenous peptide inhibitors such as p21 in the p53 non-functional cells, and thereby deregulated CDK2 activity. Publications reporting activation of p53 in p53 WT cells by CDK inhibitors, and hence greater cellular sensitivity, have largely been generated with non-CDK2 selective agents, for example roscovitine and flavopiridol.[25] In this setting, p53 activation can be due to both direct effects, e.g. altered p53 phosphorylation, and indirect effects, e.g. MDM2 suppression, and the lack of selectivity of the inhibitors complicates the interpretation of the data.

The greater sensitivity of the Rb WT SKUT-1B cells in comparison to the Rb null SKUT-1 cells is also consistent with CDK2 as a target for NU6102. Given that Rb is a key CDK2 substrate, and Rb-phosphorylation is a key event in cell cycle progression, the reduced activity of NU6102 in the Rb-null cell line might be expected. However, it is important to note that the magnitude of the differential activity of NU6102 against p53 non-functional and WT cells, and Rb WT and null cells, was modest and whether p53 or Rb status have potential roles as predictive biomarkers for CDK2 inhibitors requires validation in *in vivo* and clinical studies. Nonetheless, the p53 non-functional Rb WT SKUT-1B cell line was amongst the tumour cell lines most sensitive to NU6102 in the current study, and hence this line was used for *in vivo* investigations.

The N-acetyl prodrug of NU6102, NU6301, was rapidly converted to NU6102 both *in vitro* and *in vivo*, and displayed markedly improved aqueous solubility. The use of N-acetylation as a water-solubilising prodrug strategy has previously been described for sulphonamides including cyclin-dependent kinase inhibitors,[26,27] and the current study provides further evidence of the utility of this approach. NU6301 at a dose of 120 mg/kg was well tolerated and gave rise to tumour NU6102 concentrations and inhibition of Rb phosphorylation that was consistent with that observed with growth inhibitory concentrations of NU6102 *in vitro*. Consistent with the pharmacokinetic and pharmacodynamic data, 8 and 12 hourly dosing of 120 mg/kg NU6301, equivalent to 100 mg/kg NU6102, produced statistically significant growth delay in SKUT-1B human tumour xenografts. Further evaluation of the compound in tumour models where selective susceptibility to CDK2 inhibition might be anticipated, for example *MYC* or *MYCN* amplified tumours,[11–13] is warranted.

In conclusion, a prodrug of the CDK2 inhibitor NU6102 has been developed and characterised *in vitro* and *in vivo*, and the compound will allow the extension on mechanistic studies, such as those performed with NU6102,[28] and facilitate the further evaluation of CDK2 as a target in cancer.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.04.008.

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