Combined PI3K and CDK2 inhibition induces cell death and enhances *in vivo* antitumour activity in colorectal cancer

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Background: The phosphatidylinositol-3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathway is commonly deregulated in human cancer, hence many PI3K and mTOR inhibitors have been developed and have now reached clinical trials. Similarly, CDKs have been investigated as cancer drug targets.

Methods: We have synthesised and characterised a series of 6-aminopyrimidines identified from a kinase screen that inhibit PI3K and/or mTOR and/or CDK2. Kinase inhibition, tumour cell growth, cell cycle distribution, cytotoxicity and signalling experiments were undertaken in HCT116 and HT29 colorectal cancer cell lines, and in vivo HT29 efficacy studies.

Results: 2,6-Diaminopyrimidines with an O^4-cyclohexylmethyl substituent and a C-5-nitroso or cyano group (**1,2,5**) induced cell cycle phase alterations and were growth inhibitory ($GI_{50} < 20 \mu$M). Compound **1**, but not **2** or **5**, potently inhibits CDK2 ($IC_{50} = 0.1$ nM) as well as PI3K, and was cytotoxic at growth inhibitory concentrations. Consistent with kinase inhibition data, compound **1** reduced phospho-Rb and phospho-rS6 at GI_{50} concentrations. Combination of NU6102 (CDK2 inhibitor) and pictilisib (GDC-0941; pan-PI3K inhibitor) resulted in synergistic growth inhibition, and enhanced cytotoxicity in HT29 cells in vitro and HT29 tumour growth inhibition in vivo.

Conclusions: These studies identified a novel series of mixed CDK2/PI3K inhibitors and demonstrate that dual targeting of CDK2 and PI3K can result in enhanced antitumour activity.

Insights into the molecular pathology of cancer have resulted in the development of targeted approaches for cancer therapy. The phosphatidylinositol-3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathway is one of the most commonly deregulated pathways in human cancer in which signals are transduced via a cascade of lipid and serine/threonine kinases resulting in diverse effects on proliferation, apoptosis, protein translation, DNA repair, survival and transformation (for review see (Mayer and Arteaga, 2015)). Activation of receptor tyrosine kinases (RTKs) stimulates the PI3K pathway via adapter proteins, and PI3K phosphorylates phosphoinositide-3,4-biphosphate (PIP_2) to generate PIP_3 that activates AKT(PKB). AKT has many functions including roles in cell migration, apoptosis and glucose uptake, and the activation of mTOR via downregulation of the TSC1/Rheb complex. mTOR in a complex with Raptor (to form mTORC1) signals via p70S6 kinase and 4EBP1 to regulate the translation of proteins and cell growth. mTOR also complexes with Rictor (to form mTORC2) to activate AKT. Negative feedback occurs at many nodes in the PI3K/mTOR cascade, most notably by PTEN and by S6 kinase-mediated regulation of RTK signalling, for example, by downregulation of IRS-1 (Harrington *et al*, 2005).

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Activating mutations in the PIK3CA (p110α) isoform of PI3 kinase are observed in a wide range of tumours including breast, colon, glioblastoma and ovarian cancers (Cancer Genome Atlas Research Network, 2008; Stemke-Hale *et al*, 2008; Mayer and Arteaga, 2015), and inactivation of the tumour suppressor protein PTEN has been documented in, among others, glioblastoma, breast cancer, prostate cancer and melanoma (Cairns *et al*, 1997; Garcia *et al*, 1999; Celebi *et al*, 2000; Cancer Genome Atlas Research Network, 2008). More rarely AKT mutations or amplifications have been detected in colorectal, head and neck, pancreatic and breast tumours, and large a proportion of cancers have activation of mTORC1 as demonstrated by increased phosphorylation of proteins such as S6 kinase, 4EBP1, ribosomal S6 protein and mTOR itself (reviewed in (Menon and Manning, 2008)).

Considerable effort has been focussed on the development of PI3K/mTOR pathway inhibitors, many of which have now reached clinical trials (Yap *et al*, 2015). Rapamycin, discovered as an antifungal agent from *Streptomyces hygroscopicus*, was the first mTOR inhibitor characterised and a number of the trial candidates are rapamycin analogues (rapalogues). Rapamycin binds to FK-binding protein 12 and therefore only inhibits the mTORC1 complex and not mTORC2. Rapamycin has been approved for the treatment of advanced renal cell carcinoma and, following promising Phase III trials, the rapalogues temsirolimus and everolimus have been approved for renal cell carcinoma and soft tissue/bone sarcomas, respectively (Chiarini *et al*, 2015). In addition, compounds are in clinical trials that block both mTORC1 and mTORC2 by competitive inhibition at the ATP-binding site (Chiarini *et al*, 2015).

ATP competitive inhibitors of PI3 kinase have also entered clinical trials (Chiarini *et al*, 2015). These compounds mainly target the p110α isoform of PI3K, but some have been developed that have selectivity for other isoforms, for example, idelalisib inhibits p110δ but not α or β (Lannutti *et al*, 2011). Compounds such as dactolisib target PI3K and mTOR at similar potency, and others such as pictilisib are pan-PI3K inhibitors (Brachmann *et al*, 2009). A review of published early clinical trial data indicates that PI3K inhibitors are well tolerated and as single agents they can induce disease stabilisation, and in some cases regression (Brachmann *et al*, 2009; Engelman, 2009). Preclinical data suggest that combinations of PI3K inhibitors with other targeted therapies, for example, MEK inhibitors (Engelman *et al*, 2008; Haagensen *et al*, 2012; Haagensen *et al*, 2013), can result in significant synergism and clinical trials to explore such combinations are ongoing.

Cyclin-dependent kinases (CDKs) regulate cell cycle progression and transcription via sequential interaction with specific cyclins. In some cancers defects in the expression of CDKs and their regulatory proteins, for example, loss of CDK inhibitors or upregulation of cyclin expression, leads to aberrant regulation of the cell cycle (Satyanarayana and Kaldis, 2009). CDK2 has an important role in the G1 to S-phase transition and also has a role in S-phase progression. In particular, phosphorylation of Retinoblastoma protein (Rb) by cyclin D/CDK4 and 6 complexes, followed by the cyclin E/CDK2 complex, inactivates Rb releasing E2F and thereby promoting S-phase entry and progression (Harbour *et al*, 1999; Aleem *et al*, 2004). Numerous small molecule inhibitors of CDKs have been evaluated in clinical trials (Shapiro, 2006; Asghar *et al*, 2015), some of which are pan-CDK inhibitors such as flavopiridol, whereas other compounds are more selective. Preliminary clinical data with CDK inhibitors suggests they are generally well tolerated but rarely induce marked anti-proliferative toxicity or tumour regressions when given as single agents (Shapiro, 2006; Asghar *et al*, 2015). Thus, with the exception of flavopiridol for the treatment of chronic lymphocytic leukaemia (Lin *et al*, 2009), where the transcriptional kinase CDK9 is thought to be the primary target, and palbociclib for the management of ER-positive HER2-negative breast cancer, where CDK4/6 is the

target (Rocca *et al*, 2014), it has yet to be determined whether CDK-specific or pan-CDK inhibitors are preferable (Shapiro, 2006), or indeed if this compound class generally possesses widespread antitumour activity.

In a counter-screen of compounds designed to inhibit CDK2 we identified a number of molecules that were also mTOR and/or PI3 kinase inhibitors. These compounds provided an opportunity to evaluate the impact of simultaneous modulation of cell cycle and survival/growth signalling, via dual CDK and PI3K/mTOR inhibition, which revealed that potent CDK2 inhibition combined with PI3K inhibition resulted in cell cycle and growth inhibition, and cytotoxicity. *In vitro* results with a dual CDK/PI3K inhibitor were recapitulated using a combination of a selective CDK2 inhibitor (NU6102) and a pan-PI3K inhibitor (pictilisib), and extended to *in vivo* studies that showed enhanced tumour growth inhibition with the drug combination.

MATERIALS AND METHODS

Materials. Dactolisib and pictilisib were purchased from Stratech Scientific (Suffolk, UK) and rapamycin from Merck (Nottingham, UK). Anti phospho-4EBP1 (Cat# 2855S), 4EBP1 (Cat# 9644), phospho-AKT (Cat# 4060S), AKT (Cat# 4691S), phospho-ribosomal S6 (Cat# 4858S) and ribosomal S6 (Cat# 2217S) antibodies were purchased from NEB (Hitchin, UK). Anti phospho-Rb (Cat# 44–582 G) was supplied by Invitrogen (Paisley, UK) and anti-Rb (Cat# 554136) by BD Biosciences (Oxford, UK). All other reagents were analytical grade where available and supplied by Sigma (Gillingham, UK).

Synthesis of compounds. The following compounds were synthesised as previously described: **1** (Sayle *et al*, 2003), **2** (Arris *et al*, 2000), **3** and **4** (Mesguiche *et al*, 2003), **5** and **6** (Marchetti *et al*, 2010). **7** and **8** were synthesised as described in Supplementary Material. NU6102 (4-[[6-(cyclohexylmethoxy)-9H-purin-2-yl]amino]-benzenesulfonamide) was synthesised as previously described (Davies *et al*, 2002).

Enzyme assays. PI3Ks (Condliffe *et al*, 2005) and CDK2 (Arris *et al*, 2000) assays were performed as previously described. The inhibition of mTOR kinase activity at $10\,\mu M$ ATP was measured by TR-FRET according to the manufacturer's instructions (http://tools.invitrogen.com/Content/SFS/lanthascreen/PV4753%20FRAP1%20Assay%20Validation.pdf).

Cell culture. HCT116 and HT29 cells were obtained from ATCC (Teddington, UK), and grown in DMEM supplemented with 10% (v/v) fetal bovine serum, $100\,\text{units ml}^{-1}$ penicillin, $0.1\,\text{mg ml}^{-1}$ streptomycin and 2 mM L-glutamine.

Cell growth inhibition assays. Cells were trypsinised and plated in 96-well plates at a seeding density of 1000 cells per well. Twenty-four hours later cells were incubated for 72 h with 0.001–100 μM of each inhibitor dissolved in DMSO with a final DMSO concentration of 1% (v/v). Cells were fixed with 50% (w/v) TCA and stained with 0.4% (w/v) sulforhodamine B (SRB) for 30 min before washing with 1% (v/v) acetic acid. SRB was solubilised with 10 mM Tris pH 10.5, the absorbance read at 570 nM, and cell growth expressed as % of the growth of cells exposed to 1% (v/v) DMSO. The concentration of compound that resulted in 50% growth inhibition (GI_{50}) was calculated by interpolation from the growth inhibition/concentration curves using GraphPad Prism software (La Jolla, USA). Combination growth inhibition studies of NU6102 and pictilisib were performed as previously described (Haagensen *et al*, 2012).

Clonogenic assays. Cells were seeded in six-well plates at a density of 1×10^5 per well and allowed to adhere overnight. The GI_{50}

concentration from the SRB assay for the pyrimidine compounds **1–8** was added to the cells for 8 or 72 h. Cells were washed twice with PBS before the addition of standard growth medium. Following incubation to allow colony (>50 cells) formation, cells were fixed with Carnoy's solution and stained with 0.4% (w/v) crystal violet solution for 1 h. Colonies were washed with 1% (v/v) acetic acid and counted using an Oxford Optronix Colcount (Oxford, UK). Single agent and combination cytotoxicity studies with NU6102 and pictilisib at 1 or 10 μM were performed using the same method with cells being exposed to drugs for 72 h before plating out for colony formation. Statistical analyses were performed using ANOVA.

Flow cytometry. Cells were seeded in six-well plates at a density of 4×10^5 per well and allowed to adhere overnight. Incubation of the cells with the GI$_{50}$ concentration of each compound for up to 24 h, was followed by washing with PBS and cell removal with trypsin. Trypsinised cells were centrifuged, washed with PBS and fixed with 75% ethanol/25% PBS (v/v) overnight. Pellets were rehydrated with PBS and stained using 1 mg ml^{-1} RNase and 400 mg ml^{-1} propidium iodide for 4 h, and analysed using a Becton Dickinson FACSscan and Cellquest software (BD biosciences) with data evaluation using WinMdi software (http://facs.scripps.edu/software.html) and ANOVA statistical analysis.

Western blotting. Cells were seeded in six-well plates at a density of 4×10^5 per well and allowed to adhere overnight. Cells were incubated with 0.5, 5 or 50 μM **1** or **5** for 4, 8 or 24 h before being washed and extracted with Phosphosafe buffer (Merck). Protein concentrations were determined by bicinchoninic acid assay (Thermo Scientific, Cramlington, UK) and 30 μg of total protein for each sample was loaded into the wells of a 4–20% tris-glycine Novex gel (Invitrogen). Proteins were resolved at 120 V for $\sim$2 h before being transferred to Hybond-C nitrocellulose (GE Health-care, Chalfont St Giles, UK). Membranes were probed with the appropriate dilution of antibody overnight and, after washing, the appropriate secondary antibody used at a dilution of 1 : 1000, followed by analysis using Supersignal West Dura enhanced chemiluminescence substrate (Thermo Scientific).

In vivo studies. All of the in vivo experiments were reviewed and approved by the institutional animal welfare committee, and performed according to the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (second edition) and national law. CD-1 nude mice (Charles River, Ramsgate, Kent, UK) were implanted with 1×10^7 HT29 cells, in a mixture of Matrigel/RPMI cell culture media (1 : 1 v/v), subcutaneously on the right flank. Tumours were allowed to develop until they were palpable ($\sim 0.5 \times 0.5$ cm, 10–12 days after implantation) and randomised into four treatment groups (six animals per group).

Mice were treated with vehicle (control), NU6301 120 mg kg^{-1} i.p. (a water soluble prodrug of NU6102 (Thomas et al, 2011); 120 mg /kg^{-1} NU6301 being equivalent to 100 mg kg^{-1} NU6102) twice daily, 100 mg kg^{-1} pictilisib p.o. once daily, or the combination of twice daily NU6301 (120 mg kg^{-1} i.p.) and once daily pictilisib (100 mg kg^{-1} p.o.) for 10 days. Tumour volume was calculated from two-dimensional calliper measurements using the equation $a^2 \times b/2$, where a is the smallest measurement and b the largest. 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 in accordance with the formula: RTV = tumour volume on day of observation/tumour volume on day 0. Statistical analyses of the effects of drug treatment was undertaken using the Mann–Whitney test.

RESULTS

The structures of the compounds tested are shown in Supplementary Figure 1 and their activity as inhibitors of CDK2, the four PI3K isoforms and mTOR in purified kinase assays is shown in Table 1; ranked in order of potency as inhibitors of CDK2. Sulphonamide **1** is an extremely potent inhibitor of CDK2 (IC$_{50}$ 0.1 nM) and a low μM inhibitor of all of the PI3K isoforms with the exception of PI3Kβ. However, **1** did not inhibit mTOR at the highest concentration tested (20 μM). The parent compound in this series, **2**, inhibited CDK2 and all of the PI3K isoforms at similar concentrations (1–10 μM), but was somewhat less potent against mTOR. The cyano compound **7** has IC$_{50}$ values in the range 10–20 μM for CDK2 and the PI3Kα, δ and γ isozymes, but like **1** is less active against PI3Kβ and mTOR. The nitroso compound **6** is similar to **7**, although somewhat less active against CDK2 and more active against PI3Ks α, δ and γ. The aldehyde **4** and cyano compound **5** are similar to **6**, although **4** is distinct in having an IC$_{50}$ for mTOR of 15 μM, vs $<5\%$ inhibition for **6** and **5** at 20 μM. Also, **5** is less active than **6** and **4** against CDK2. Importantly, the O^4-(4-chlorobenzyl) compound **8** did not inhibit CDK2 but is an inhibitor of all four PI3K isozymes and mTOR, whereas **3** is only active against mTOR (IC$_{50}$ 13 μM).

The cell growth inhibitory activity of each compound was tested using the SRB assay in HCT116 and HT29 cells. GI$_{50}$ values were calculated from the mean of three separate assays for each compound, and are given in Table 2. Rapamycin, dactolisib (NVP-BEZ235), pictilisib (GDC-0941; pictrelisib) and NU6102 were used as positive controls for mTOR inhibition, dual PI3K and mTOR inhibition, pan-PI3K inhibition and CDK2 inhibition, respectively. Compounds **1**, **2** and **5** were equipotent in both cell lines, similar in potency to rapamycin, but markedly less potent than the dual PI3K/mTOR inhibitor dactolisib. Inhibitors **4**, **7** and **8** also had similar GI$_{50}$ values, but were 5–10-fold less potent at inhibiting cell

Table 1. Inhibition of CDK2, PI3K and mTOR kinases by 6-aminopyrimidines

Compound	CDK2	PI3Kα	PI3kβ	PI3Kδ	PI3Kγ	mTOR
1	0.0001 ± 0.00003	3.6, 6.2	>20	3.2, 7.5	3.7, 6.8	>20
2	2.2 ± 0.5	1.3, 2.7	5.7, 11	0.7 , 1.8	1.5, 2.1	19
7	9.6, 13	9.9, 12	>20	12, 14	18, 20	>20
6	7.5, 22	2.9, 4.4	>20	5.0, 6.5	2.9, 7.1	>20
4	22, 24	5.4, 11	>20	2.9, 5.6	1.7, 5.3	15
5	60 ± 10	4.5, 7.7	18, 23	6.9, 5.9	7.5, 12	>20
8	$>>10$	2.5, 6.2	4.3, 13	0.5, 1.1	1.5, 2.0	4.9
3	$>>10$	>20	>20	>20	>20	13

Abbreviations: mTOR = mammalian target of rapamycin; PI3K = phosphatidylinositol-3-kinase. Values are the IC$_{50}$ (μM) or the highest concentration tested (μM), and are the mean $\pm$ s.d. or individual determinations. For chemical structures see Supplementary Figure 1.

growth than **1**, **2** and **5**. Growth inhibition induced by **3** and **6** did not reach 50% at 100 μM, inducing a maximum of 35% reduction in growth at 100 μM in HCT116 cells.

The effects of the compounds on cell cycle phase distribution at GI_{50} concentrations, or at 100 μM for **6** and **3**, in asynchronous cultures were evaluated by flow cytometry in HCT116 cells (Figure 1A). An increase in the G1 population was observed, of varying magnitude, with **2**, **4** and **5** producing greater G1 phase accumulation than rapamycin or dactolisib (NVP-BEZ235). No effect on cell cycle phase distribution was observed with 100 μM **3**. Compound **1** was unique in inducing the formation of a sub-G1

fraction, indicative of apoptosis (Figure 1). Further analysis of the effect of **1** on the cell cycle indicated that the sub-G1 fraction became apparent from 3 h onwards in HCT116 cells (Figure 1B). The increase in the sub-G1 fraction was significant after 3 h ($P = 0.013$) and remained significant up to 8 h ($P < 0.002$). The G1 fraction was reduced after 4 h exposure to **1**, but was only significant at the 10% level ($P < 0.09$), whereas the S-phase fraction was significantly reduced at 4 h ($P < 0.016$). The increase in the G2/M phase fraction was significant at both 4 and 6 h ($P < 0.011$). In HT29 cells, **1** induced a time-dependent increase in the G2/M fraction, with a sub-G1 fraction becoming apparent from 6 h onwards (Figure 1C), which was concentration-dependent at 24 h (Supplementary Figure 2). Although a sub-G1 fraction was apparent at 6 and 8 h, this was not significant ($P = 0.183$ at 8 h); whereas the G1 fraction was significantly reduced after 4 h ($P < 0.002$). The S-phase fraction in HT29 cells was significantly reduced after 6 h ($P < 0.04$) treatment with **1**, and the G2/M fraction was significantly increased after 4 h ($P < 0.002$).

To determine whether the sub-G1 fraction observed following treatment with **1** reflected cytotoxicity, clonogenic survival assays were performed in HCT116 and HT29 cells following an 8 h exposure. Compound **1** reduced the clonogenic survival of cells in a concentration-dependent manner in both HCT116 and HT29 cells (Figure 2A and B), with 50% inhibition of colony formation at 5 μM. Consistent with the lack of a sub-G1 peak in flow cytometry experiments, the other pyrimidine inhibitors were not cytotoxic in either cell line even after 72 h exposure (HCT116 cells; Figure 2C).

To explore the contribution of CDK2 inhibition to the cytotoxicity of **1**, the effects of **1** and **5** on target protein phosphorylation were examined in HCT116 (Figure 3) and HT29 (Supplementary Figure 3) cells following 4, 8 and 24 h exposure. Compound **5** was selected as being equipotent to **1** against PI3Ks and mTOR, with similar growth inhibitory potency in both cell lines but 600 000-fold less active against CDK2

| Table 2. Colorectal tumour cell growth inhibition by 6-aminopyrimidines		
Compound	GI_{50} HCT116 (μM)	GI_{50} HT29 (μM)
1	4.3 ± 2.6	12 ± 3
2	6.2 ± 1.8	12 ± 4
7	73 ± 28	> 100 μM (30%)
6	> 100 μM (35%)	> 100 μM (1%)
4	64 ± 18	44 ± 5
5	6.7 ± 1.8	14 ± 6
8	52 ± 11	59 ± 20
3	> 100 μM (30%)	> 100 μM (17%)
Dactolisib (NVP-BEZ235)	0.016 ± 0.008	0.014 ± 0.001
Rapamycin	2.3 ± 1.5	4.2 ± 1.4
NU6102	5.7 ± 0.4	13.3 ± 3.4
Pictilisib (GDC-0941)	1.7 ± 0.3	0.25 ± 0.13
Values are the mean GI_{50} ± s.d., or the highest concentration tested and the % growth inhibition observed at that concentration.		
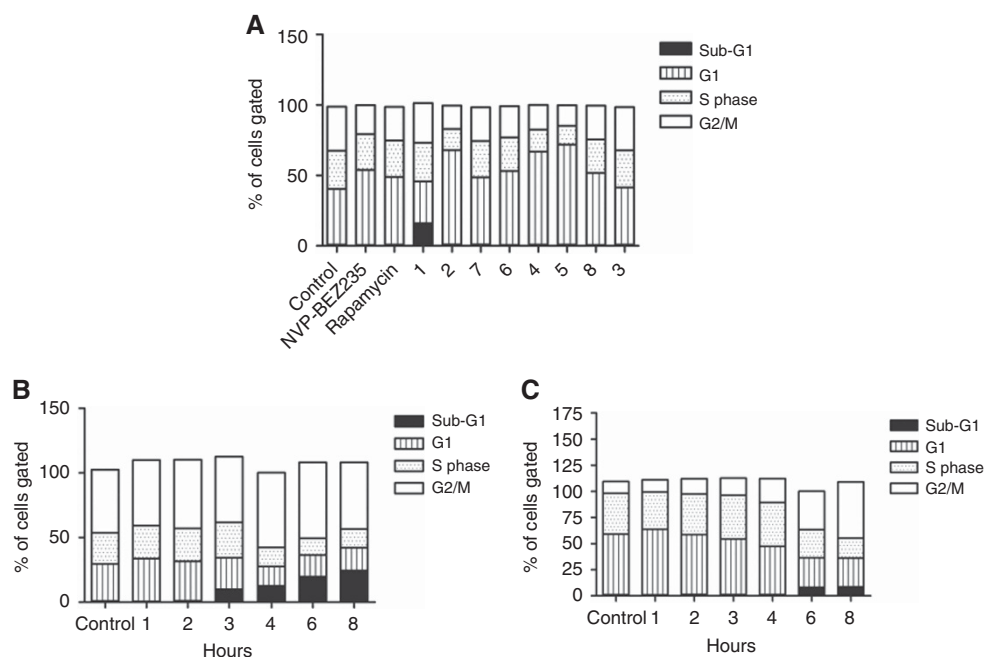
Figure 1. Cell cycle phase analysis following exposure to 6-aminopyrimidine inhibitors measured by flow cytometry. (**A**) HCT116 cells were treated with GI_{50} concentrations of inhibitors (or 100 μM, Table 2) for 24 h then fixed, stained with propidium iodide and analysed by flow cytometry. Cell cycle phase distribution was analysed by WinMDI software, and the mean cell cycle fraction of cells gated into each phase of the cell cycle shown. (**B**) HCT116 cells were treated with the GI_{50} concentration of **1** for up to 8 h and fixed at time points indicated before being stained with propidium iodide and analysed by flow cytometry. (**C**) HT29 cells were treated with the GI_{50} concentration of **1** for up to 8 h and fixed at the time points shown before being stained with propidium iodide and analysed by flow cytometry. Data are representative of duplicate experiments.
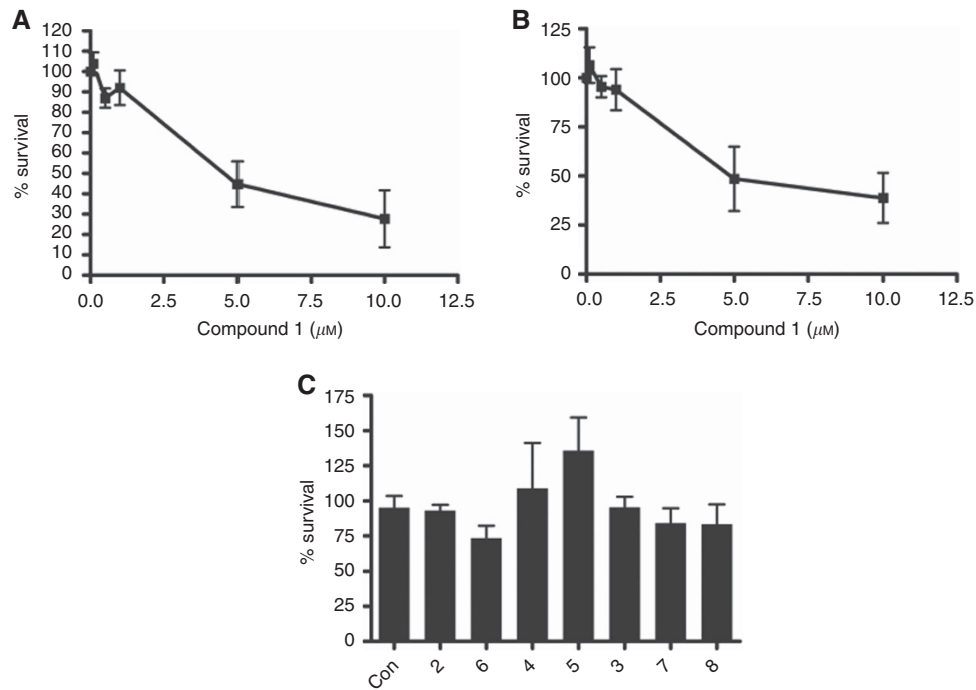
Figure 2. Effects of 6-aminopyrimidine inhibitors on the clonogenic survival of HCT116 and HT29 colorectal cancer cells. (A) HCT116 cells were treated with increasing concentrations of 1 for 8 h. The medium was replaced with normal growth medium and the cells allowed to form colonies for 1 week. Colonies were stained with 0.4% (v/v) crystal violet and counted. (B) HT29 cells were treated as described in (A). (C) HCT116 cells were treated with the GI_{50} concentration (or $100\,\mu M$, Table 2) of the 6-aminopyrimidine inhibitors for 72 h before being re-plated for analysis of colony formation as described in (A). All experiments were performed in triplicate and the data are the mean ± s.d.
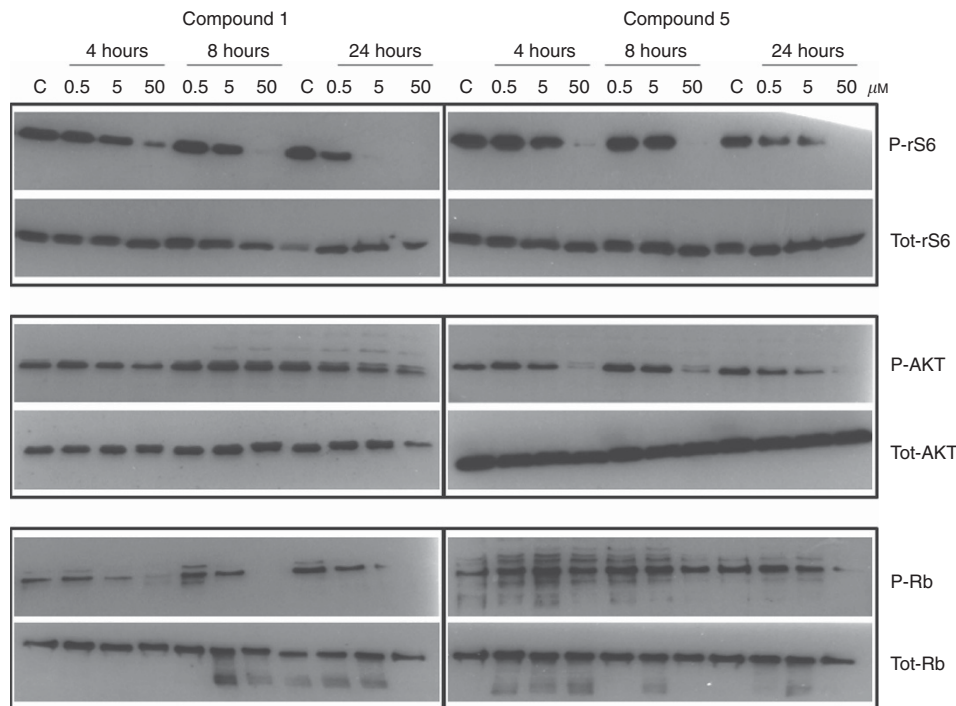

Figure 3. Effects of compounds 1 and 5 on the phosphorylation of rS6, AKT and Rb in HCT116 cells. HCT116 cells were treated with 1 or 5 for 4, 8 or 24 h at 0.5, 5 or $50\,\mu M$. Protein was extracted and $30\,\mu g$ resolved on a 4–20% (w/v) tris/glycine SDS–PAGE. Immuno-blotted membranes were probed with total and phospho-rS6, AKT and Rb antibodies and the western blots shown are representative of at least three experiments.

(Tables 1 and 2). Compound 1 induced concentration-dependent inhibition of Rb phosphorylation in HCT116 cells, with inhibition at a GI_{50} concentration of $5\,\mu M$ being observed at all time points, and at 24 h in HT29 cells. In contrast, the effect of 5 on Rb phosphorylation was less pronounced and only clearly observed after 24 h exposure to $50\,\mu M$. Compounds 1 and 5 had similar effects on rS6 phosphorylation in both cell lines, and only 5 consistently reduced AKT phosphorylation at $50\,\mu M$.
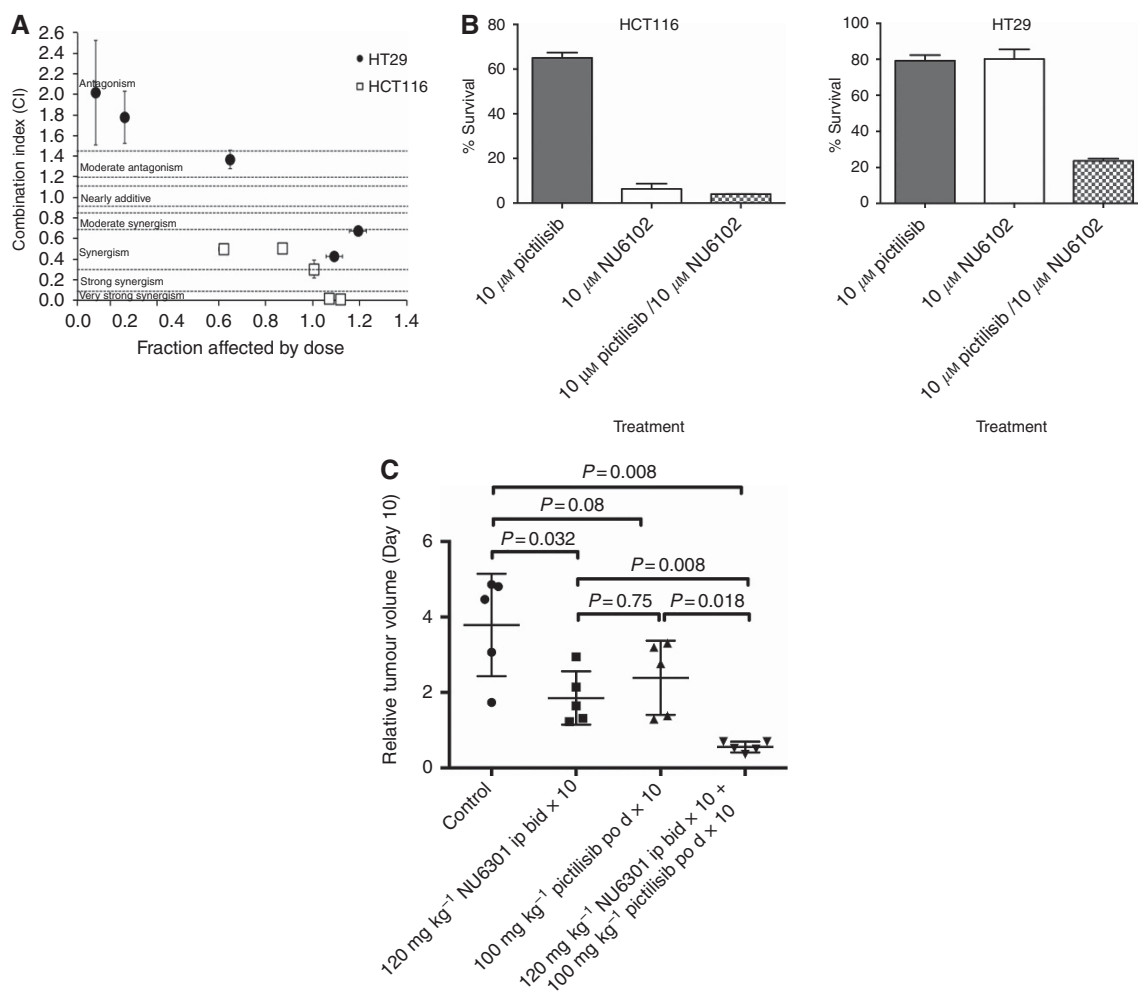
Figure 4. The impact of combined CDK2 and PI3K inhibition on colorectal tumour cell growth and survival *in vitro*, and HT29 tumour growth *in vivo*. (A) HCT116 or HT29 cells were exposed to combinations of NU6102 and pictilisib at fixed ratios ($0.25\times$, $0.5\times$, $1\times$, $2\times$ and $4\times$) of their respective GI_{50} concentrations (NU6102: HCT116 5.7 μM, HT29 13 μM; pictilisib: HCT116 1.7 μM, HT29 0.25 μM) and cell growth inhibition evaluated by median effect analysis. (B) Cytotoxicity of 1 μM or 10 μM NU6102 and pictilisib alone and in combination in HCT116 and HT29 colorectal tumour cell lines. (C) Efficacy of the NU6102 prodrug NU6301 and pictilisib, alone and in combination, in HT29 human colorectal tumour xenograft bearing mice. Mice were treated with vehicle (control), NU6301 120 mg kg^{-1} i.p. (equivalent to 100 mg kg^{-1} NU6102) twice daily, 100 mg kg^{-1} pictilisib p.o. once daily, or the combination of twice daily NU6301 (120 mg kg^{-1} i.p.) and once daily pictilisib (100 mg kg^{-1} p.o.) for 10 days. Data are the relative tumour volume (RTV) at the end of treatment (day 10) and *P*-values are the statistical differences between the groups as indicated by the Mann–Whitney test.

The results obtained with compound **1** in comparison with the other compounds suggested that dual CDK2 and PI3K inhibition resulted in cytotoxicity, and to confirm this suggestion studies were performed with the potent CDK2 inhibitor NU6102 (4-[[6-(cyclohexylmethoxy)-9H-purin-2-yl]amino]-benzenesulfonamide) (Davies *et al*, 2002) in combination with the pan-PI3K inhibitor pictilisib. As shown in Figure 4A, the combination of NU6102 and pictilisib was synergistic in growth inhibition studies in both the HCT116 and HT29 cell lines. Furthermore, the combination of the two drugs at 10 μM was cytotoxic to HT29 cells, which was significantly enhanced compared with either compound alone at this concentration (Figure 4B; $P \leqslant 0.0001$). However, in the HCT116 cell line, there was only a significant increase in cytotoxicity with the combination compared to pictilisib alone ($P \leqslant 0.0001$), as NU6102 was cytotoxic as a single agent (Figure 4B).

An *in vivo* tumour growth inhibition study was performed using the HT29 model treated for 10 days with the NU6102 prodrug NU6301 at 120 mg kg^{-1} twice each day (equivalent to 100 mg kg^{-1} NU6102), pictilisib (GDC-0941) at 100 mg kg^{-1} per day or the combination of the two drugs at these doses, which

were based on *in vivo* pharmacodynamic data from earlier studies (Thomas *et al*, 2011; Haagensen *et al*, 2013). Compound **1** was not suitable for *in vivo* studies as it contains a 5-nitroso group, which is notoriously metabolically labile and potentially reactive. As shown in Figure 4C, RTV in mice treated with the combination of NU6301 and pictilisib (GDC-0941) were significantly lower in comparison with either of the drugs alone ($P = 0.008$ and $P = 0.018$, respectively, Mann–Whitney test) at the end of treatment (day 10). There was no tumour growth in the combination group while on treatment, whereas single-agent treatment at the doses used only generated growth delays, which were not significant in the case of NU6301 alone (time to RTV4 *vs* control – $P = 0.13$, Mann–Whitney test), and not highly significant in the case of pictilisib (GDC-0941; time to RTV4 *vs* control – $P = 0.026$, Mann–Whitney test). In contrast, growth delay was highly significant for the combination therapy (time to RTV4 *vs* control – $P = 0.0043$, Mann–Whitney test). Although a formal toxicology study was not performed, the combination treatment was well tolerated with no consistent impact of single-agent or combination drug treatment on body weights or clinical condition.

DISCUSSION

Inhibitors of mTOR, PI3K and CDK2 are currently in various stages of clinical development. Extensive preclinical studies indicate that combinations of targeted agents may demonstrate greater antitumour activity and selectivity than single agents, the combination of PI3K and MEK inhibitors being an excellent example (Engelman *et al*, 2008; Haagensen *et al*, 2012; Haagensen *et al*, 2013). Counter-screening of compounds synthesised to target CDK2 identified a number of pyrimidines that were able to inhibit PI3K and/or mTOR, and we evaluated inhibitors with a range of potencies against CDK2, PI3K and mTOR for their effects on cell growth, cell cycle phase distribution and cytotoxicity.

In the two colorectal cancer cell lines studied, HCT116 and HT29, three of the compounds had GI_{50} values of $<20 \mu M$: **1**, **2** and **5**. The wide range of potencies of these compounds as inhibitors of CDK2 (0.1, 2200 and 60 000 nM, respectively) and the lack of activity against mTOR of two of the compounds ($<5\%$ inhibition at $20 \mu M$: **1** and **5**), yet similar potency for PI3K inhibition, is not inconsistent with growth inhibition owing to activity against the latter targets. The reduced growth inhibitory properties on **4**, **6**, **7** and **8**, despite similar potency against PI3Ks to **1**, **2** and **5**, may be due to reduced cellular uptake resulting from the major structural changes at the 4-position (replacement of the cyclohexylmethyl group in **6**, **7**, **8**) or 5-position (replacement of the nitroso group with formyl in **4**). In this latter regard, removal of the 5-nitroso group (**3**) resulted in complete loss of activity against CDK2 and PI3Ks; however, interestingly, modest mTOR inhibition was retained. Nevertheless, despite the mTOR inhibitory activity of **3**, the compound did not inhibit cell growth. The reason for the lack of CDK2 inhibition by **3** can be explained by the absence of an intra-molecular hydrogen bond as in **1** (i.e., between the 5-nitroso and the 6-amino group), which is critical for maintaining the correct orientation of the 6-amino group for hydrogen bonding to Glu-81 of CDK2 (Arris *et al*, 2000). A similar effect may explain the lack of activity of **3** against PI3Ks, although there are no structural data to support this contention.

When asynchronous cell cultures were treated with GI_{50} concentrations of the growth inhibitory compounds the predominant effect was an accumulation of cells in the G1 phase of the cell cycle. Both rapamycin and dactolisib (NVP-BEZ235) also caused a G1 arrest at their GI_{50} concentrations, an effect that has been previously reported with PI3K and/or mTOR inhibitors (Mita *et al*, 2003; Serra *et al*, 2008). In contrast to the other growth inhibitory compounds, **1** initially either had no effect on the fraction of cells in the G1 phase (HCT116) or reduced the size of the G1 fraction (HT29), before rapidly ($\leqslant 6 h$) inducing a sub-G1 population in both cell lines indicative of apoptosis, although only significantly in the HCT116 cells. The impact of the apoptosis induced by **1** was revealed by clonogenic cytotoxicity studies where the compound was shown to be cytotoxic to both HCT116 and HT29 cells following exposure for 8 h at growth inhibitory concentrations. In marked contrast, exposure of HCT116 cells for 72 h to all of the other pyrimidines failed to induce significant cytotoxicity. Together, these cellular data suggest that the potent CDK2 inhibitory activity of **1** contributes to the cytotoxic activity of the compound.

To evaluate whether the relative potencies of compounds against purified kinases reflected their activity in a cellular context, western blotting experiments were performed with **1** and the non-CDK2 inhibitory control compound, **5**. As expected, **1** inhibited the phosphorylation of the CDK2 substrate Rb at earlier time points and at lower concentrations than **5**, and these data are consistent with the hypothesis that the apoptosis and cytotoxicity induced by **1** is related to CDK2 inhibition. Interestingly, despite good inhibition of rS6 phosphorylation, compound **1** did not inhibit the phosphorylation of AKT, which is likely to reflect phosphorylation at this site by other pathways, for example, IKK has recently been implicated in promoting AKT phosphorylation at Ser473 (Dan *et al*, 2016).

To confirm that the cytotoxicity observed with **1** was due to dual CDK2 and PI3K inhibition, combination studies were performed with the potent and selective CDK2 inhibitor NU6102 in combination with the pan-PI3K inhibitor pictilisib. In growth inhibition studies, a synergistic as opposed to an additive interaction was observed, particularly in the HCT116 cell line, and in cytotoxicity studies combination of the two inhibitors was markedly more cytotoxic in HT29 cells than either compound alone. Importantly, the superior activity of the combination of the CDK2 and the PI3K inhibitor was recapitulated in an *in vivo* antitumour study without any marked increase in host toxicity, as evaluated by body weight changes and clinical observations. The specificity of NU6102 as a CDK2 inhibitor has been well-established (Davies *et al*, 2002) and confirmed subsequently in a study using CDK2 WT and KO MEFs (Thomas *et al*, 2011). At the concentrations and doses of NU6102 used in the current study ($10 \mu M$ *in vitro* and $100 \, mg \, kg^{-1}$ *in vivo*), these previous studies have demonstrated CDK2 specificity (Davies *et al*, 2002; Thomas *et al*, 2011). Pictilisib is a very widely used and well-characterised PI3K inhibitor, and was used at doses and concentrations in line with previous published studies (Haagensen *et al*, 2012; Haagensen *et al*, 2013). Consequently, these tool compounds can be used to show that dual CDK2 and PI3K inhibition may represent an approach to improving antitumour selectivity with these two classes of targeted agents.

Mechanistically, the potential of dual CDK2 and PI3K pathway inhibition has been highlighted by a number of studies. Initially, Maddika *et al*, 2008 demonstrated that nuclear translocation of AKT results in phosphorylation of CDK2/cyclin A, leading to cytoplasmic redistribution of CDK2/cyclin A, which was required for S to G2-M cell cycle phase progression. Subsequently, a landmark paper (Liu *et al*, 2014) reported that phosphorylation of AKT by CDK2/cyclinA2 at S477/T479 was a critical priming event that precedes AKT activation by phosphorylation at the canonical S473 locus, and that by this mechanism CDK2/cyclinA1 activity is a prerequisite for the oncogenic activity of AKT and hence more generally the PI3K pathway. These recent mechanistic insights into the interplay between CDK2 and PI3K signalling provide a rationale for the improved activity of dual CDK2 and PI3K inhibition described in the current paper. Furthermore, the effects of the dual pyrimidine-based inhibitor **1** and combined treatment with the selective CDK2 antagonist NU6102 and the pan-PI3K inhibitor pictilisib described here are consistent with the results using alternative small molecule CDK and PI3K inhibitors, and siRNA (Cheng *et al*, 2012). In this latter study, increased apoptosis *in vitro* and enhanced tumour growth delay *in vivo* were observed when both CDK2 and PI3K, specifically PI3Kα, were modulated by chemical and/or molecular genetic means.

In summary, we have synthesised and characterised a series of 6-aminopyrimidines identified from a kinase screen that are inhibitors of PI3K and/or mTOR and/or CDK2. In HCT116 and HT29 cell lines, PI3K inhibition is associated with cell growth inhibition, regardless of the activity against CDK2. Likewise, the level of mTOR inhibition does not influence cell growth inhibitory potency. Importantly, the mixed CDK2 and PI3K inhibitor **1** induces apoptosis and is cytotoxic, suggesting that dual CDK2/PI3K targeting may be preferential to inhibition of either enzyme alone. Mechanistically, the recent identification of direct interactions between CDK2 and AKT, the proximal downstream target of PI3K signalling, provides a rationale for the simultaneous targeting of the CDK2 and the PI3K pathway in order to achieve maximal antitumour effects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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