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The Aurora kinase inhibitor CCT137690 downregulates MYCN and sensitizes *MYCN*-amplified neuroblastoma *in vivo*

Amir Faisal¹, Lynsey Vaughan², Vassilios Bavetsias¹, Chongbo Sun¹, Butrus Atrash¹, Sian Avery¹, Yann Jamin³, Simon P. Robinson³, Paul Workman¹, Julian Blagg¹, Florence I. Raynaud¹, Suzanne A. Eccles¹, Louis Chesler², and Spiros Linardopoulos^{1,4}

¹Cancer Research UK, Cancer Therapeutics Unit, The Institute of Cancer Research, Sutton, UK

²Paediatric Oncology, The Institute of Cancer Research, Sutton, UK

³Cancer Research UK and EPSRC Cancer Imaging Centre, The Institute of Cancer Research and Royal Marsden NHS Trust, Sutton, Surrey, UK

⁴Breakthrough Breast Cancer Research Centre, London, UK

Abstract

The Aurora kinases regulate key stages of mitosis including centrosome maturation, spindle assembly, chromosome segregation and cytokinesis. Aurora A and B overexpression has also been associated with various human cancers and as such, they have been extensively studied as novel anti-mitotic drug targets. Here we characterise the Aurora kinase inhibitor CCT137690, a highly selective, orally bioavailable imidazo[4,5-*b*]pyridine derivative that inhibits Aurora A and B kinases with low nanomolar IC₅₀ values in both biochemical and cellular assays and exhibits anti-proliferative activity against a wide range of human solid tumour cell lines. CCT137690 efficiently inhibits histone H3 and TACC3 phosphorylation (Aurora B and Aurora A substrates, respectively) in HCT116 and HeLa cells. Continuous exposure of tumour cells to the inhibitor causes multipolar spindle formation, chromosome misalignment, polyploidy and apoptosis. This is accompanied by p53/p21/BAX induction, thymidine kinase 1 (TK1) downregulation and PARP cleavage. Furthermore, CCT137690 treatment of *MYCN*-amplified neuroblastoma cell lines inhibits cell proliferation and decreases MYCN protein expression. Importantly, in a transgenic mouse model of neuroblastoma (TH-*MYCN*) that overexpresses MYCN protein and is predisposed to spontaneous neuroblastoma formation, this compound significantly inhibits tumour growth. The potent preclinical activity of CCT137690 suggests that this inhibitor may benefit patients with *MYCN* amplified neuroblastoma.

Corresponding Author: Spiros Linardopoulos, Team Leader, Cancer Research UK, Cancer Therapeutics Unit, The Institute of Cancer Research, 15 Cotswold Road, Sutton, SM2 5NG and Breakthrough Breast Cancer Research Centre 237, Fulham Road, London, SW3 6JB, UK. TEL: +44 20 7153 5341, FAX: +44 20 7143 5340, spiros.linardopoulos@icr.ac.uk.

Disclosure of Potential Conflicts of Interest

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Keywords

Aurora; Neuroblastoma; Inhibitor; MYCN

Introduction

Neuroblastoma is the most common extra-cranial solid tumor of childhood, accounting for approximately 10% of pediatric tumours and affects more than ten thousand children worldwide each year. It originates from the sympathetic nervous system and is characterized by heterogeneous pathological and clinical presentation. Stage 4 neuroblastoma represents approximately 50% of cases with metastatic dissemination at diagnosis and its prognosis is poor. Therefore, novel therapeutic strategies are urgently needed to improve the prognosis of neuroblastoma patients. Amplification of the *MYCN* gene is associated with an aggressive form of neuroblastoma that results in a particularly poor clinical outcome (1).

Downregulation of MYCN protein by targeting with siRNA, or alternatively, destabilizing the protein using an inhibitor of the upstream PI3K signalling pathway, has been shown as an effective preclinical therapy for neuroblastoma (2, 3). Furthermore, an association between MYCN and Aurora kinases has also been reported; a synthetic lethal screen identified AURKA as a gene required for the growth of *MYCN*-amplified neuroblastoma cell lines. In addition, Aurora A was shown to stabilize MYCN protein through its physical interaction with MYCN and the E3 ubiquitin ligase FBXW7 (4). A recent study using MLN8237, a small molecule inhibitor of Aurora A kinase, has also shown efficacy against neuroblastoma in preclinical models (5). Therefore, inhibition of the Aurora kinases may be an effective strategy to treat *MYCN*-amplified neuroblastoma.

The Aurora kinases are a family of highly conserved serine/threonine kinases that are important for faithful transition through mitosis (6-8). Their activity and protein expression are cell cycle regulated, peaking during mitosis to orchestrate important mitotic processes including centrosome maturation, chromosome alignment, chromosome segregation and cytokinesis (6, 8, 9). In humans, the Aurora kinase family consists of three members; Aurora A, Aurora B and Aurora C, that each share a conserved C-terminal catalytic domain but differ in their sub-cellular localization, substrate specificity and function during mitosis (9, 10). At the onset of mitosis, Aurora A binds to TPX2 and is activated by autophosphorylation at threonine 288. Once activated, Aurora A phosphorylates multiple substrates that regulate centrosome maturation and mitotic spindle assembly (11, 12). Aurora B is part of the chromosomal passenger complex (CPC) along with inner centromere protein (INCENP), Survivin and Borealin. Aurora B is activated through binding and phosphorylation of INCENP in a positive feedback mechanism (13). Since the phosphorylation of histone H3 at serine10 (S10) and serine28 (S28) by Aurora B is required for chromosome condensation (14), inhibition of S10 phosphorylation has been widely used as a biomarker for Aurora B inhibition *in vitro* and *in vivo* (15). Aurora C is also a chromosomal passenger protein but unlike Aurora A and B, its expression is restricted to testes (16).

The amplification and over-expression of Aurora A and Aurora B has been reported in many human tumours, including breast, colon, neuroblastoma, pancreatic and ovarian cancers, with high Aurora A expression levels being associated with poor prognosis and genomic instability (17, 18). These properties have made the Aurora kinases attractive anti-mitotic drug targets and as such a number of Aurora kinase inhibitors are currently being evaluated pre-clinically and in clinical trials (16). We have recently identified CCT137690 as a potent and highly selective Aurora kinase inhibitor, described its mode of binding to Aurora A kinase and shown its *in vivo* efficacy in xenografts in mice using human cancer cell lines (19). In addition to inhibiting the Aurora kinases, we have recently identified CCT137690 as a potent inhibitor of oncogenic FLT3 and demonstrated its *in vitro* and *in vivo* activity in FLT3-ITD positive acute myeloid leukaemia (20).

Aurora kinase inhibitors therefore represent new agents for the treatment of a variety of cancers including neuroblastoma, particularly when associated with *MYCN* gene-amplification. Here we report an extensive cellular characterisation and the potent preclinical activity of CCT137690 in *MYCN*-amplified neuroblastoma *in vitro* and *in vivo*.

Material and Methods

Plasmids and reagents

Myc-tagged Aurora A cloned in pCR2.1 vector has been previously described (21). pCMV expression vector for human TACC3 was a kind gift from Dr Fenni Gergley. Synthesis of CCT137690, an imidazo[4,5-b]pyridine derivative, has been described elsewhere (19). Rabbit polyclonal antibodies against transforming acidic coiled-coil 3 (TACC3) phosphorylated at S558 were raised against phospho-peptide TADLDI[p]TEINK (Eurogentech). Other antibodies used were, phospho-Aurora A (T288), total Aurora A, BAX, cleaved PARP, P-GSK3 β , (Cell signalling), phospho-histone H3 (Millipore) total histone H3 (Abcam), p21, p53, myc, MYCN (Santa Cruz), tubulin (Sigma) and GAPDH (Chemicon International).

Cell culture, transfections and immunoblotting

All cell lines were purchased from the American Type Culture Collection (ATCC) except the SHEP that was obtained from the University of California at San Francisco (San Francisco, CA, USA) Cell Culture Facility (San Francisco, CA) and KELLY that was purchased from the Health Protection Agency Culture Collections. All cell lines were grown in their recommended culture medium, supplemented with 10% FBS at 37°C in 5% CO₂ and passaged for less than 6 months prior to replacement from early passage frozen stocks. Cells were transfected at approximately 80% confluency with the plasmids indicated using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. For immunoblotting, cells were collected either in NP-40 lysis buffer (120 mM NaCl, 50 mM Tris HCl pH 7.5, 1% NP-40 supplemented with phosphatase and protease inhibitors), or 2X LDS sample buffer (Invitrogen). Equal amount of proteins were resolved by 4-12% Bis-Tris NuPAGE gels (Invitrogen), transferred to Nitrocellulose (Whatman) membranes, and immunoblotted with specific antibodies. Cells were regularly screened for Mycoplasma, using a PCR-based assay (VenorGem; Minerva Biolabs).

Cell proliferation assays

Cell proliferation assays were performed by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Sigma) as described elsewhere (22). Briefly, cells were plated in 96-well plates at 3,000 cells per well and were treated with a range of 0 to 25 mol/L of CCT137690 for 72 h. The absorbance was measured at 570 nm using the Wallac VICTOR2 1420 Multilabel Counter (PerkinElmer).

Cell cycle and immunofluorescence analysis

Cell cycle analysis was performed as described previously (22), briefly cells were treated with different concentration of CCT137690 for 24, 48 and 72 hours, harvested with 5 mmol/L EDTA-PBS and fixed in 85% ice-cold ethanol. Fixed cells were then stained with propidium iodide (Sigma)/RNase solution for 30 min at 37°C, and analyzed on Beckman Coulter Cytomics FC500. For immunofluorescence, cells were grown on coverslips in 24 well tissue culture plates, treated with CCT137690 for 4, 24 or 48 hours and fixed with 4% formaldehyde for 15 min at room temperature or with ice-cold methanol for 15 min at -20°C (for phospho-Aurora-T288). Cells were then permeabilized and blocked with 2% BSA in PBS containing 0.1% Triton X-100 before incubation with primary antibodies for 1h at room temperature (1:100 dilutions in 2% BSA). Cells were washed three times with PBS and incubated with appropriate Alexa 488 or 568 antibodies for 45 minutes (1:1000 dilution in 2% BSA) and washed three times with PBS. To visualize nuclei, TO-PRO-3 iodide (Invitrogen; 1:10000) was added during the last wash. Coverslips were mounted on glass slides with Mowiol (Polysciences) and fluorescence was visualized with a Leica SP1confocal microscope.

In vivo efficacy studies

Animals were randomized into two groups, group 1: treatment with 100 mg/kg CCT137690 n=4 or group 2: vehicle control n=4. Treatment was administered via oral gavage twice daily. Tumour volumes were measured at day 0, 3 (48 hours after treatment started), 7 and 10 using ¹H MRI. ¹H MRI was performed on a 7T Bruker horizontal bore microimaging system using a 3 cm birdcage coil. Anaesthesia was induced by an intraperitoneal injection of a combination of fentanyl citrate (0.315 mg/ml) plus fluanisone (10 mg/ml) (Hypnorm, Janssen Pharmaceutical, Oxford, UK), midazolam (5 mg/ml) (Roche, Welwyn Garden City, UK) and water (1:1:2). Anatomical T2-weighted images were acquired from twenty contiguous 1 mm thick coronal slices through the mouse abdomen with a 240 µm inplane resolution (RARE sequence, NEX=4, TE_{eff}=36 ms, TR=4500 ms, turbo factor=8, 1 mm thick contiguous, matrix=128×128). Tumour volume was measured using segmentation from regions of interest drawn on every slice-containing tumour using in-house software Imageview (written under IDL programming platform, ITT, Boulder, Colorado, USA). Significance analysis was done using the Student's t test. CCT137690 was quantified in extracted plasma and tumour samples by HPLC with tandem mass spectrometry using reverse phase gradient elution chromatography and multiple reaction monitoring. All animal studies were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and national guidelines (23)

Results

CCT137690, a selective Aurora kinase inhibitor, potently inhibits proliferation of human tumour cell lines

Our lead optimization studies to develop a potent, highly selective and orally bioavailable Aurora kinase inhibitor resulted in discovery of CCT137690 (Figure 1A), an imidazo[4,5-b]pyridine that inhibits Aurora A, B and C kinases with IC₅₀ values of 0.015, 0.025 and 0.019 μ M respectively (19). CCT137690 is highly selective and inhibited the activity of 3 additional kinases namely, FLT3 (IC₅₀ 0.0025 μ M), FGFR1 and VEGFR above 80% at 1 μ M in a panel of 94 kinases (19). Here we evaluated the anti-proliferative activity of CCT137690 against a wide number of human tumour cell lines using an MTT assay. CCT137690 effectively inhibited the growth of human tumour cell lines of different origins with GI₅₀ (growth inhibition by 50%) values ranging from 0.005 μ M to 0.47 μ M (Figure 1B). Within this panel of cell lines we did not find an obvious relationship between sensitivity to growth inhibition and the protein levels of the Aurora kinases. Differences in sensitivity might rather be associated with different genetic backgrounds of the cell lines, for example, lack of proteins associated with Aurora kinases (22).

CCT137690 inhibits both Aurora A and B in cells

We next investigated the ability of CCT137690 to inhibit Aurora A autophosphorylation at T288 and histone H3 phosphorylation at S10 (an Aurora B substrate) in HeLa cells. Both the autophosphorylation of ectopically expressed Aurora A and phosphorylation of histone H3 (pHH3) were efficiently inhibited by CCT137690 in a dose dependent manner (Figure 2A), with complete inhibition being seen for both P-T288 and pHH3 at 0.5 μ M of CCT137690. The blots were quantified by densitometry (Image J software, NIH) and the IC₅₀ values were calculated to be 0.025 μ M and 0.093 μ M for P-T288 and pHH3 respectively. The phosphorylation of transforming acidic coiled-coil (TACC3) by Aurora A at S558 is required for its localization at centrosomes and proximal microtubules and has been used as a biomarker for Aurora A inhibition in cells (24, 25). We have generated and characterized an antibody against TACC3 phosphorylated at S558 (Suppl. Figure 1). To test whether CCT137690 inhibits TACC3 phosphorylation, cells overexpressing TACC3 and Aurora A were treated with increasing concentrations of CCT137690 for 2 hours following overnight treatment with nocodazole for mitotic arrest. As shown in figure 2B, TACC3 phosphorylation at S558 was inhibited in a dose dependent manner. In addition, the inhibition of histone H3 phosphorylation at S10, derived from the same cell lysate, is also shown (Figure 2B). We next analysed the inhibition of histone H3 S10 phosphorylation in nocodazole-arrested HeLa cells by immunofluorescence. Histone H3 phosphorylation at S10 in mitotic cells was completely inhibited after 24 hours treatment with 0.5 μ M CCT137690 (Figure 2C). CCT137690 also inhibited autophosphorylation of endogenous Aurora A in mitotic cells as examined by immunofluorescence (Figure 2D).

CCT137690 induces polyploidy, mitotic aberrations and apoptosis in tumour cells

Aurora B plays an important role in cytokinesis and its inhibition results in accumulation of cells with 4N DNA content (22, 26). We tested whether CCT137690 induces a similar phenotype in HCT116 cells. As shown in figure 3A, treatment of HCT116 cells with 0.5 or 1

μM CCT137690 for 24 hours induced accumulation of cells with 4N and 8N DNA content, indicating cytokinesis failure and induction of polyploidy. Moreover, following 48 and 72 hours of treatment with CCT137690, cells with 16N DNA content can also be observed (Figure 3A). A similar induction of 4N cell population by CCT137690 was also observed in HeLa cells (Suppl. Figure 2). To test the effects of CCT137690 on mitosis, HeLa cells were treated with 0.5 and $1\mu\text{M}$ of CCT137690 for 24 and 48 hours, then analysed by immunofluorescence. As shown in figure 3B, treatment with CCT137690 for 24 hours induced multipolar spindle formation and misaligned chromosomes. In addition, when using 0.5 μM of the inhibitor, monopolar spindles were also observed in the cells, consistent with Aurora A inhibition. Extension of the treatment to 48 hours resulted in multiple aberrant spindles, indicative of polyploid cells. Continuous exposure of HCT116 cells to CCT137690 for up to 72 hours resulted in increased levels of p53, the pro-apoptotic protein BAX and PARP cleavage (Figure 3C; Suppl. Figure 3). Treatment of HCT116 cells with 0.5 μM of CCT137690 for 24 hours upregulated p53 and p21 and downregulated TK1 (Suppl. Figure 4), confirming previously reported modulation of these biomarkers by Aurora inhibition that may be used in a non-invasive PET imaging to monitor the activity of Aurora inhibitors *in vivo* (22).

CCT137690 inhibits growth of neuroblastoma cell lines with *MYCN* amplification

Aurora A has been shown to stabilize MYCN by altering FBXW7-induced ubiquitin-mediated proteolytic degradation (4). Therefore, we first tested whether CCT137690 specifically sensitises the growth of *MYCN*-amplified human neuroblastoma cell lines. A panel of neuroblastoma cell lines expressing different levels of MYCN was treated with CCT137690. Neuroblastoma cell lines with increased levels of MYCN (KELLY, SH-SY5Y, IMR32 and SHEP with stable expression of exogenous wild type MYCN) showed higher sensitivity to CCT137690 than those with low or no-detectable MYCN expression (SK-N-SH, SHEP; Table I). Treatment of KELLY, a *MYCN* amplified neuroblastoma cell line, with CCT137690 resulted in a reduction of both p-T288 Aurora A and pHH3 at nanomolar doses, indicative of Aurora A and Aurora B inhibition respectively (Figure 4A). Similar inhibition of pHH3 by CCT137690 was observed in four cell lines with different levels of MYCN (Suppl. Figure 5) indicating that the difference in sensitivity of these cell lines to CCT137690 is not due to differential inhibition of Aurora kinases but possibly due to differences in MYCN levels. To assess the effect of CCT137690 on MYCN protein levels, KELLY cells were treated with different concentrations of CCT137690 for 6 or 24 hours, stimulated with 50 ng/ml IGF1 prior to harvesting and analysed by immunoblotting. As shown in Figure 4B, treatment with CCT137690 at both 6 (left panel) and 24 hours (right panel) resulted in a decreased steady state of MYCN levels at 3- and 6-fold the GI_{50} dose of CCT137690. Reduction of MYCN levels also correlated very closely with decreasing levels of GSK3 β phosphorylation, as indicated by Ser9 phosphorylation of GSK3 β (Figure 4C). The phosphorylation of GSK3 β at Ser9 by Aurora A kinase is inactivating (27, 28). Active GSK3 β phosphorylates MYCN at Thr58, which is required for its recognition by the E3 ubiquitin ligase FBXW7, resulting in MYCN ubiquitination and proteolytic degradation (29). To test whether CCT137690-mediated downregulation of MYCN and inhibition of GSK3 β phosphorylation at Ser9 was due to Aurora A inhibition, KELLY cells overexpressing Aurora A were treated with CCT137690. Overexpression of Aurora A in

KELLY cells increased both MYCN protein levels and GSK3 β phosphorylation at S9 compared to the vector control (Figure 4C). Aurora A inhibition by CCT137690 reduced both MYCN expression and Ser9 GSK3 β phosphorylation (Figure 4C). This data indicates that the downregulation of MYCN is due to Aurora A inhibition by CCT137690.

CCT137690 inhibits growth of MYCN-driven transgenic tumours *in vivo*

We next tested whether inhibition of Aurora kinases reduces the growth of *MYCN*-amplified neuroblastomas *in vivo*. We conducted a pre-clinical trial testing the ability of CCT137690 to impact on established tumours in a *MYCN*-driven transgenic mouse model (TH-*MYCN*); TH-*MYCN* mice are predisposed to spontaneous formation of neuroblastoma through tissue-specific overexpression of MYCN within the murine neural crest (30). Importantly, this model recapitulates the major clinic-pathologic features of high-risk, *MYCN*-amplified neuroblastoma and was previously used to establish activity of LY294002, cyclophosphamide and anti-angiogenic agents (3, 30). Animals were treated with either a vehicle control or CCT137690 at a dose of 100 mg/kg twice daily for 10 days. Tumour volumes were measured at day 0, 3, 7 and 10 using ^1H MRI. In animals treated with CCT137690, a growth inhibition was observed as early as day 3 and was highly significant ($p=0.00469$) when compared with vehicle control (Figure 5A). Moreover, continuous treatment with the inhibitor showed significant ($p<0.05$) tumour growth inhibition at day 7 and day 10 (Figure 5A). CCT137690 levels within plasma and tumours were well above the $\text{GI}_{50}/\text{IC}_{50}$ for target inhibition, as confirmed by mass spectrometry (Figure 5B). Taken together this data confirms, both *in vitro* and *in vivo*, the potency of Aurora kinase inhibitors in *MYCN* driven neuroblastoma.

Discussion

The Aurora kinases are serine/threonine kinases that play essential roles during mitosis (6, 10). In addition, the Aurora kinases are amplified and overexpressed in a wide variety of cancers and their upregulation often correlates with poor prognosis. Therefore, they have been extensively studied as potential therapeutic targets in solid tumours and hematopoietic cancers. (6, 17, 18, 31). In this study we further characterized the novel Aurora kinase inhibitor CCT137690 (19). The compound showed considerable selectivity to Aurora kinases among a panel of kinases tested and inhibited the growth of a wide range of human tumour cell lines. This sensitivity might be associated with the inhibition of all Aurora kinases, in particular Aurora-A and Aurora-B, in addition to a different genetic background among the cell lines. Our results, consistent with previous studies, showed that inhibition of Aurora kinases by CCT137690 resulted in stabilisation of p53, p21 and the pro-apoptotic protein BAX in tumour cells. This led to PARP cleavage and apoptosis. Our data also confirmed that CCT137690 downregulates thymidine kinase 1 (TK1). Non-invasive [^{18}F] FLT PET imaging, as previously reported, may therefore be used to measure the activity of CCT137690 *in vivo* (22).

Aurora A and B have been shown to phosphorylate the centrosomal protein TACC3 at Ser558 and histone H3 at Ser10, respectively (24, 32). Consistent with this, we showed that CCT137690 potently inhibits the phosphorylation of both substrates in tumour cells.

CCT137690 treatment of human colon cancer HCT116 cells caused cytokinesis failure that leads to abnormal mitotic spindles and cells with 4N DNA content. The observed abnormal mitotic spindles and multinucleation of tumour cells treated with CCT137690 resembles the phenotype induced in cells by inhibiting Aurora A and Aurora B using siRNA or other chemical inhibitors (33, 34).

Neuroblastoma is one of the deadliest solid tumours in children (35). Up to 60% of patients present with widely metastatic disease (more often bone and bone marrow metastases), with a poor long-term survival rate <10% (35) despite intensive treatments including surgery, chemotherapy and irradiation. Thus, a search for novel, more potent and less toxic treatments of neuroblastoma is currently underway. The particularly aggressive subtype of neuroblastoma characterized by *MYCN*-amplification, represents approximately 20% of total cases. Aurora A has been previously suggested to cause *MYCN* stabilization in human neuroblastoma by inhibiting FBXW7-induced ubiquitination and degradation (4). It was suggested that stabilization of *MYCN* was due to the direct interaction with Aurora A that inhibited its proteosomal degradation in a kinase-independent manner. In more detail, Aurora A was shown to promote the synthesis of non-K48-linked ubiquitin chains, thereby inhibiting FBXW7-induced ubiquitination and degradation of *MYCN*. There is however growing evidence, as shown in this study and in other recent studies (5, 36), that neuroblastoma cells are highly sensitive to Aurora kinase inhibitors both *in vitro* and *in vivo*. Whether Aurora kinase inhibitors organize Aurora A protein in a structural conformation that destabilizes its interaction with *MYCN* still remains to be answered. It would be interesting to study if similar mechanisms control the expression levels of other members of the *MYC* family, such as c-Myc, which are also targets for degradation by the ubiquitin ligase FBXW7. In a previous study, we have shown that inhibition of Aurora kinases by the small molecule inhibitor CCT137202 reduced the transcriptional levels of c-Myc, as shown by expression array analysis (22). Therefore, there are at least two mechanisms through which the Aurora kinases control the expression of the *MYC* genes, one at the transcriptional and another at the post-translational level. These observations strongly suggest that Aurora inhibitors would significantly enhance the treatment of Myc-dependent cancers. Further studies using selective inhibitors towards Aurora A, will be required to determine whether the observed destabilization of *MYCN* is primarily due to its ubiquitination and proteolytic degradation, stimulated by Aurora A inhibition, or whether this occurs by alternative mechanisms.

Here in this study, using the novel Aurora kinase inhibitor CCT137690, we identified the Aurora kinases as critical protein targets in neuroblastoma cell lines and tumours. We showed that *MYCN*-amplified neuroblastoma cell lines are more sensitive to CCT137690. CCT137690 treatment reduced the levels of *MYCN* and the phosphorylation/inactivation of the GSK3 β kinase. Furthermore, CCT137690-mediated downregulation of *MYCN* protein and GSK3 β phosphorylation is specifically due to Aurora A inhibition. Importantly, CCT137690 dramatically reduces neuroblastoma tumour mass in *MYCN* transgenic mice, when used as a single agent. In summary, CCT137690 is a highly selective, orally available Aurora kinase inhibitor with potent *in vitro* and *in vivo* efficacy in *MYCN*-amplified neuroblastomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A **B**

Cell Line	CCT137690 GI ₅₀ μ M
SW48	0.005
T84	0.14
SW620	0.15
Ls174T	0.16
SW403	0.18
SW948	0.19
HCT116	0.22
DLD1	0.26
Colo320	0.36
PC/JW2	0.45
LOVO	0.47
A2780	0.35
HeLa	0.14

Figure 1.

CCT137690 is a potent pan-Aurora kinase inhibitor with anti-proliferative activity against number of human tumour cell lines. A. Structure of CCT137690. B. Anti-proliferative activity of CCT137690 in a variety of human tumour cell lines as measured by a 3-day MTT assay.

Figure 2.

CCT137690 potently inhibits Aurora A and B in HeLa cells. A. HeLa cells expressing Myc-Aurora A were treated with different concentrations of CCT137690 for 2 hours and analysed by immunoblotting using P-T288 and pHH3 S10 antibodies. Blots were quantified and the IC₅₀ values calculated. B. HeLa cells were co-transfected with Aurora A and TACC3, treated overnight with nocodazole followed by treatment with different concentrations of CCT137690 for 2 hours. TACC3 phosphorylation at S558 and histone H3 phosphorylation at S10 were analysed by immunoblotting. Total TACC3 and total histone H3 were used as loading control. C. Immunofluorescence of HeLa cells treated with 50ng/ml nocodazole overnight, followed by treatment with CCT137690 for 24 hours. Cells were fixed and stained with pHH3 S10 antibody. D. Immunofluorescence of HeLa cells treated with CCT137690 for 4 hours, fixed and stained with P-T288 antibody. DAPI staining (blue) indicates the DNA content (Bar = 50 μm).

Figure 3.

CCT13760 induces aberrant mitosis, polyploidy and apoptosis. **A.** HCT116 cells were treated with different concentrations of CCT137690 for 24, 48 and 72 hours, fixed, stained and analysed by flow cytometry. The DNA content is indicated by arrows. **B.** HeLa cells were treated with indicated concentrations of CCT137690 for 24 and 48 hours. Cells were fixed, stained and analysed by immunofluorescence using Aurora A (green) and alpha-tubulin antibodies (red). DAPI staining (blue) indicates the DNA content (Bar = 50 μm). **C.** HCT116 cells were treated with indicated concentrations of CCT137690 for 24, 48 and 72 hours. Cell lysates were analysed by immunoblotting using cleaved PARP, p53 and BAX antibodies. GAPDH was used as a loading control.

Figure 4.

CCT137690 reduces MYCN levels and GSK3 β phosphorylation in the KELLY neuroblastoma cell line. A. Cells were treated overnight with 50ng/ml nocodazole followed by 2 hours treatment with indicated concentrations of CCT13690. Phosphorylation of Aurora A at T288 and histone H3 at S10 was analysed by immunoblotting using phospho-specific antibodies. Total Aurora A and total histone H3 were used as loading controls. B. KELLY cells were treated with the indicated concentrations of CCT137690 for 6 or 24 hours and stimulated with 50 ng/ml of IGF1 for 30 minutes. The level of MYCN protein and phosphorylation of GSK3 β at S9 were determined by immunoblotting using phospho-specific antibodies. β -actin was used as loading control. C. KELLY cells were transfected with empty vector or plasmids constructs carrying Aurora A. 16 hours after transfection, cells were treated with DMSO control or indicated concentration of CCT137690 for 24 hours. Cell lysates were analysed for MYCN, GSK3 β (S9), Aurora A (exogenous and endogenous) and GAPDH by immunoblotting.

Figure 5.

CCT137690 inhibits growth of MYCN-induced neuroblastoma *in vivo*. A. Tumour-bearing animals transgenic for TH-MYCN were treated using either 100 mg/kg CCT137690 (n=4) or vehicle (n=4). Treatment was twice daily via oral gavage for 10 days, at which time the animals were sacrificed. Data = mean $\pm$ s.e.m., n=4. *p<0.05, ***p<0.005, unpaired Student's t-test. ^a n=3, mouse with the larger tumour enrolled in vehicle cohort died before day 7. B. Pharmacokinetic analysis of plasma and tumours from TH-MYCN mice treated with CCT137690.

Table ICellular GI₅₀ of CCT137690 in human neuroblastoma cell lines with different MYCN expression levels

Cell Line	CCT137690 GI ₅₀ (μM) (Mean, n=3) 72 hrs
KELLY (MYCN Amp ^{high})	0.33
IMR32 (MYCN Amp ^{high})	0.38
SHEP WT (SHEP with stable expression of WT MYCN)	0.39
SH-SY5Y (High MYCN protein expression)	0.92
SK-N-SH (Low MYCN protein expression)	4.93
SHEP (No detectable levels of MYCN protein)	9.21

N.B. Both SH-SY5Y and SHEP cell lines derived from parental SK-N-SH