Inhibition of the aurora kinases suppresses *in vitro* NT2-D1 cell growth and tumorigenicity

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Abstract

The aurora kinase family members, Aurora-A, -B, and -C (listed as AURKA, AURKB and AURKC respectively in the HUGO Database), are serine/threonine kinases involved in the regulation of chromosome segregation and cytokinesis, and alterations in their expression are associated with malignant cell transformation and genomic instability. Deregulation of the expression of the aurora kinases has been shown to occur also in testicular germ cell tumors (TGCTs) identifying them as putative anticancer therapeutic targets. We here evaluated the *in vitro* effects of MK-0457, an aurora kinases inhibitor, on cell proliferation, cell cycle, ploidy, apoptosis, and tumorigenicity on the TGCT-derived cell line NT2-D1. Treatment with MK-0457 inhibited cell proliferation in a time- and dose-dependent manner, with $IC_{50} = 17.2 \pm 3.3$ nM. MK-0457 did not affect the expression of the three aurora kinases, but prevented their ability to phosphorylate substrates relevant to

the mitotic progression. Time-lapse experiments demonstrated that MK-0457-treated cells entered mitosis but were unable to complete it, presenting after short time the typical features of apoptotic cells. Cytofluorimetric analysis confirmed that the treatment with MK-0457 for 6 h induced NT2-D1 cells accumulation in the G₂/M phase of the cell cycle and the subsequent appearance of sub-G₀ nuclei. The latter result was further supported by the detection of caspase-3 activation following 24-h treatment with the inhibitor. Finally, MK-0457 prevented the capability of the NT2-D1 cells to form colonies in soft agar. In conclusion, the above findings demonstrate that inhibition of aurora kinase activity is effective in reducing *in vitro* growth and tumorigenicity of NT2-D1 cells, and indicate its potential therapeutic value for TGCT treatment.

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Introduction

The term 'germ cell tumors' refers to neoplasms originating from cells belonging to the germ cell lineage and have three main profiles characterized by different epidemiological, histological, and clinical parameters (Looijenga & Oosterhuis 1999, Lutke Holzik *et al.* 2004, von Eyben *et al.* 2005, Oosterhuis & Looijenga 2005, Horwich *et al.* 2006). They include teratomas–yolk sac tumors usually occurring over the early years of life, testicular germ cell tumors (TGCTs) forming after puberty and during adult life, and spermatocytic seminomas occurring in elderly men (von Eyben *et al.* 2005, Oosterhuis & Looijenga 2005, Horwich *et al.* 2006). Overt TGCT is thought to develop from a precursor neoplastic lesion defined as intratubular germ cell neoplasia (IGCN) and represents the most common malignancy in young adult Caucasian males (Looijenga & Oosterhuis 1999, Bray *et al.* 2006, Sokoloff *et al.* 2007).

Aneuploidy represents a hallmark of solid cancer progression, including the IGCN and TGCT (Looijenga & Oosterhuis 1999, Hanahan & Weinberg 2000, Mitelman 2000,

Horwich *et al.* 2006). Specifically, the median DNA content of seminomas is hypertriploid and that of nonseminomas is hypotriploid (Oosterhuis *et al.* 1989). Over the last decade, different mitotic proteins, showing a deregulated expression in cancer tissues, have been suggested to play a role in tumor genetic instability. Among these are the three aurora kinase family members, aurora-A, -B, and -C (listed as AURKA, AURKB and AURKC respectively in the HUGO Database), implicated in the regulation of multiple aspects of chromosome segregation and cytokinesis (Katayama *et al.* 1999, Takahashi *et al.* 2000, Tanner *et al.* 2000, Miyoshi *et al.* 2001, Nigg 2001, Sakakura *et al.* 2001, Ota *et al.* 2002, Carmena & Earnshaw 2004, Vagnarelli & Earnshaw 2004, Ulisse *et al.* 2006, 2007). Aurora-A is localized on the duplicated centrosomes and is involved in their positioning, in the recruitment of components at the pericentrosomal area and in mitotic spindle formation and stability (Nigg 2001, Carmena & Earnshaw 2004, Vagnarelli & Earnshaw 2004). Aurora-B is a chromosomal passenger protein, which is associated with chromatin at the beginning of mitosis. In a complex with other proteins as INCENP, survivin, and borealin, Aurora-B phosphorylates

proteins involved in chromosome condensation, such as histone H3 (Nigg 2001, Carmena & Earnshaw 2004, Vagnarelli & Earnshaw 2004). In metaphase, Aurora-B concentrates in the kinetochore and participates in the connections between chromatids and spindle microtubules. Moreover, during the transition from anaphase to telophase, Aurora-B plays a role in mitotic spindle dynamics and cleavage furrow, and is localized in the cytokinesis bridge. Aurora-C is also a chromosomal passenger protein, shown to be colocalized and to form complexes with Aurora-B, INCENP, and survivin in mitotic cells (Yan *et al.* 2005). In addition to the above-mentioned functions, Aurora-A is able to modulate key molecular players controlling cell proliferation and survival. Aurora-A has been shown to potentiate the oncogenic action of Ras and to modulate p53 function (Katayama *et al.* 2004, Liu *et al.* 2004, Tatsuka *et al.* 2005).

An altered expression of the aurora kinases in TGCT has been documented (Chieffi *et al.* 2004, Baldini *et al.* 2010). In particular, Chieffi *et al.* (2004) demonstrated through immunohistochemistry the expression of Aurora-B in 51% of seminoma cells and its association with the Ki-67 proliferation marker. More recently, we demonstrated, by means of quantitative RT-PCR and western blot analysis, that the expression of all three aurora kinases is deregulated in TGCT (Baldini *et al.* 2010). Moreover, the chromosome region where the Aurora-C gene maps (19q13.43) is among the chromosomal regions that are most frequently lost in TGCT (Korkola *et al.* 2008). These findings may have potential therapeutic implications. Over the last few years, specific inhibitors of aurora kinases have been identified (Harrington *et al.* 2004, Hata *et al.* 2005, Carvajal *et al.* 2006, Matthew *et al.* 2006, Manfredi *et al.* 2007). Among these inhibitors, MK-0457 (formerly VX-680) has been shown to inhibit tumor growth in xenograft models, leading to regression of leukemia, colon and pancreatic tumors at well-tolerated doses (Harrington *et al.* 2004). Thus, inhibition of the aurora kinases has provided a new tool in cancer therapy, above all for those cancers for which the available therapeutic agents are ineffective. This is also the case of a subset (20–30%) of patients affected by TGCT that do not respond to the conventional cisplatin-based chemotherapy and for whom new therapeutic approaches are needed (Nichols *et al.* 1994, Horwich *et al.* 2006, Fenner *et al.* 2008, Noel *et al.* 2008).

In the present study, we evaluated the effects of the aurora kinase inhibitor MK-0457 on cell cycle progression, proliferation, apoptosis, ploidy, and soft agar colony formation of the TGCT-derived cell line NT2-D1.

Materials and Methods

Cell line and materials

The testicular germ cell cancer-derived cell line NT2-D1 was purchased from Interlab Cell Line Collection (Genova, Italy). Bradford protein assay kit and electrophoresis reagents were

purchased from Bio-Rad Laboratories. The aurora kinases inhibitor MK-0457 was kindly provided by Merck & Co. and Vertex Pharmaceuticals Inc. (Cambridge, MA, USA). The cell proliferation reagent WST-1 was acquired from Roche Diagnostics. Monoclonal anti- β -tubulin, polyclonal anti- α -tubulin and anti- β -actin antibodies were from Sigma Chemical Co. The polyclonal anti-Aurora-C antibody was generated against a 16-amino acid peptide of the C-terminal part of Aurora-C (aa 259–275) by Eurogentec (Seraing, Belgium). The monoclonal antibodies against Aurora-A (31C1) and Aurora-B (AIM-1), and the polyclonal antibody against P-histone H3 were from Abcam (Cambridge, UK). The HRP-conjugated secondary antibodies, and the Super-Signal West Pico and West Dura chemiluminescent substrates were purchased by Thermo Fisher Scientific (Rockford, IL, USA). The secondary antibodies TRITC- and FITC-conjugated anti-rabbit and anti-mouse were from Jackson Laboratories (Bar Harbor, ME, USA). Vectashield was from Vector Laboratories (Burlingame, KS, USA).

Cell cultures

The NT2-D1 cells were cultured at 37 °C in 5% CO₂ humidified atmosphere in DMEM containing 10% FCS, 2 mM L-glutamine, and antibiotics (Andrews *et al.* 1984).

Western blot

Control and MK-0457-treated cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 150 mM sodium chloride, 1 mM EDTA, 1× protease inhibitor cocktail), sonicated, and then centrifuged at 13 000 r.p.m. for 20 min. The supernatants were recovered, and protein concentrations were determined by the Bradford assay. Aliquots of 50 µg cell protein extracts were electrophoresed on a 12.5% polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were then washed with TBST (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20), saturated with 5% low fat milk in TBST, and then incubated at 4 °C overnight with antibodies against Aurora-A (1:500), Aurora-B (1:1000), Aurora-C (1:1000), or β -actin (1:1000) in TBST. After washing, the membranes were incubated with appropriate HRP-conjugated secondary antibodies against mouse or rabbit IgG (1:1000) in TBST and developed using the chemiluminescence Super Signal kits. The Aurora kinases and β -actin immunoreactive bands were quantified by scanning densitometry, using the Molecular Analyst PC software for the Bio-Rad model 670 scanning densitometer.

Proliferation assay

The dose- and time-dependent effects of MK-0457 on NT2-D1 cell proliferation were evaluated by treating the cells respectively, with different concentrations of the inhibitor (1–500 nM) for 3 days or with the dose 200 nM for different

periods of time (1–3 days). Control cultures were performed in parallel by analogous administration of the sole vehicle dimethylsulfoxide (DMSO). Four hours before the end of incubation time, 'cell proliferating reagent WST-1' was added to the culture media, and cell viability was measured by colorimetric assay.

Flow cytometric analysis

NT2-D1 cells were cultured in the absence (plus DMSO) or in the presence of 200 nM MK-0457 for 6, 12, 24, or 72 h. Cells were collected in PBS by scraping with a rubber policeman and centrifugation at 100 *g*, for 5 min, then fixed in 70% ice-cold ethanol. Cell samples were labeled with propidium iodide and analyzed for the DNA content as previously described (Chang *et al.* 1999), using the FACScalibur Flow cytometer and CellQUEST software (BD Biosciences, San Jose, CA, USA).

Colony formation in soft agar

Petri dishes of 3.5 cm diameter were first prepared by adding 3 ml of complete medium with 0.4% soft agar. Adherent NT2-D1 cell cultures were trypsinized, centrifuged, and resuspended in a single-cell suspension of 75 000 viable cells/ml. The suspension was mixed with complete medium containing 0.4% soft agar at a ratio 1:2 and then divided into two aliquots, one of which was supplemented with MK-0457 200 nM and the other with the vehicle (DMSO). These suspensions were seeded onto the Petri dishes, 1 ml/dish, and incubated at 37 °C and 5% CO₂. Treated and nontreated cultures were observed under microscope a few hours after plating to exclude the presence of cell aggregates, and next periodically checked for colony formation. After 2 weeks, the colonies were photographed, and the acquired images were analyzed by the MetaVue software (Universal Imaging Corp., Downingtown, PA, USA), scoring those larger than 300 µm².

Time-lapse analysis

NT2-D1 cells were cultured in the absence (plus DMSO) or in the presence of 200 nM MK-0457 for 24 h under a microscope Leica DM-IRBE equipped with an incubation chamber at 37 °C and 5% CO₂. Cell pictures were performed every 5 min using the MetaVue software.

Caspase-3 assay

The NT2-D1 cells were cultured in the absence (DMSO) or in the presence of 200 nM VX-680 for 24 h. Following treatment, the cells were rinsed with PBS and collected by scraping in PBS. The cells were then used to evaluate caspase-3 activity using the caspase-3/CPP32 fluorimetric assay kit (Biovision, Mountain View, CA, USA).

Immunofluorescence

NT2-D1 cells cultured on glass coverslips were treated with 200 nM MK-0457 or the vehicle (DMSO) for 6 h, then fixed in cold methanol for 5 min, washed, and preincubated with 3% BSA in PBS for 1 h at room temperature. After three washes with PBS, the cells were incubated with the antibodies anti-Aurora-A (1:200), anti-Aurora-B (1:200), anti-Aurora-C (1:200), anti-P-histone H3 (1:1000), anti- α -tubulin (1:200), or anti- β -tubulin (1:200) for 2 h at room temperature in PBS with 1% BSA. After washing, the coverslips were incubated with TRITC- and FITC-conjugated anti-mouse or anti-rabbit antibodies (1:100) for 1 h at room temperature, and then mounted in Vectashield containing 1 µg/ml DAPI. The coverslips were observed with a microscope Leica-DMRXA.

Statistical analysis

The results were expressed as the mean $\pm$ S.E.M. of three independent experiments. The statistical significance of data was evaluated by the Student's *t*-test using the SPSS software (SPSS Inc., Chicago, IL, USA). The results were considered significantly different if the pertaining *P* values were lower than 0.05.

Results

Effects of MK-0457 on NT2-D1 cell proliferation

The effect of the aurora kinase inhibitor MK-0457 on NT2-D1 cell proliferation was evaluated on cells cultured from 6 to 72 h, in the presence of 200 nM MK-0457 or of vehicle alone (DMSO) as control. The dose of 200 nM was used in these initial experiments since it was shown to elicit maximal response on different tumor cell types *in vitro* (Harrington *et al.* 2004). The results demonstrated that MK-0457 inhibits NT2-D1 proliferation in a time-dependent manner (Fig. 1, panel A). In particular, a significant ($P < 0.01$) inhibition was detected only after 24 h of treatment and became maximal after 3 days when proliferation was reduced by more than 90%. We then evaluated the dose-dependent effects of MK-0457 on the NT2-D1 cell proliferation by treating the cells for 3 days in the presence of increasing concentrations of the inhibitor (1–500 nM). The results of these experiments, reported in Fig. 1B, showed a dose-dependent inhibition of NT2-D1 cell growth with half-maximal inhibitory concentration (IC₅₀) of 17.2 ± 3.3 nM.

Effects of MK-0457 on NT2-D1 cell apoptosis

Since the above findings showed significant cytotoxic effect of the MK-0457 on NT2-D1 cells, we evaluated by FACS analysis whether, as a consequence, the cells underwent apoptosis. Cell cultures exposed for 6 h to 200 nM MK-0457 showed an accumulation of cells in G₂/M phase (Fig. 2).

Figure 1 Effects of the MK-0457 on NT2-D1 cell proliferation. The NT2-D1-62 cells were cultured in the absence (DMSO) or in the presence of 200 nM MK-0457 for different periods of time (A) or with different concentrations of MK-0457 (1–500 nM) for 3 days (B). Four hours before the end of the incubation period, the cell proliferating reagent was added to the culture medium. Absorbance was then read at the end of the incubation period. Data reported are representative of one out of three similar experiments. Statistical significance of data was assessed by the Student's *t*-test. **P* < 0.01.

Longer exposition to MK-0457 (24 h) resulted in a significant increase in cells with sub-G₁ nuclei, which became more evident after 48 and 72 h (Fig. 2). Moreover, using microscope time-lapse analysis, we showed that control cells completed their mitosis in < 2 h (Fig. 3, panel A); in contrast, MK-0457-treated cells entered mitosis but were unable to complete it and exhibited soon after the characteristic apoptotic morphology. Moreover, a significant (*P* < 0.01) increase in caspase-3 activity was found in NT2-D1 cells after MK-0457 (200 nM) treatment for 24 h, compared with control cells (Fig. 3, panel B).

Effects of the MK-0457 on aurora kinase expression, subcellular localization, spindle formation, and histone H3 phosphorylation in NT2-D1 cells

We next investigated the alterations induced by MK-0457 on NT2-D1 mitotic structures and proteins. To ascertain whether MK-0457 effects were due to the inhibition of aurora kinase activities rather than to changes in their protein levels, we performed western blot experiments on protein extracts from control and MK-0457-treated cells (200 nM) for 6, 24, and 48 h. Densitometric analysis of western blot results of three independent experiments revealed no significant variations in Aurora-A, -B or -C proteins level at any incubation time (Fig. 4, panel A). The immunofluorescence (IF) experiments showed that centrosomal localization of Aurora-A was maintained in cells exposed to MK-0457 (200 nM) for 6 h (Fig. 4, panel B). However, the mitotic cells had aberrant spindles characterized by shorter microtubules. Aurora-B localization on the condensing

Figure 2 Effects of the MK-0457 on NT2-D1 cell apoptosis. Cells were incubated for 6, 24, 48, or 72 h with 200 nM MK-0457 or the vehicle (DMSO). At the end of the incubation time, cells were fixed and analyzed by FACS. The M1 bar identified the sub-G₁/apoptotic cell population scored, and numbers reported in the different panels represent the percentage of sub-G₀ nuclei.

chromatin during prophase was also maintained in treated cells, but, the histone H3 phosphorylation was no longer detectable (Fig. 4, panel C). In control cells, Aurora-C was solely observed on the midbody of cytokinetic cells (Fig. 4, panel D), but following MK-0457 treatment no cells in telophase could be identified.

Effects of MK-0457 on NT2-D1 cell colony formation in soft agar

We evaluated the effects of the aurora kinases inhibitor on the ability of the NT2-D1 cells to form colonies in soft agar. In these experiments, the cells were cultured either in the absence or in the presence of 200 nM MK-0457 for 2 weeks. Control cells started to form noticeable colonies after 6 days of culture, and 2 weeks later, 12.2 ± 1.0 colonies per mm^2 , with a mean area of $639 \pm 129 \mu\text{m}^2$, were scored. As shown in Fig. 5, the treatment with MK-0457 completely abolished the ability of NT2-D1 cells to form colonies in soft agar.

Discussion

Over the last decade, different experimental evidence has demonstrated that alterations in the expression of the three members of the aurora kinase family, Aurora-A, -B, and -C, may induce cell malignant transformation and genomic instability (Zhou *et al.* 1998, Nigg 2001, Carmena & Earnshaw 2004, Vagnarelli & Earnshaw 2004). These findings have led to their evaluation as a potential target for anticancer therapy

(Harrington *et al.* 2004, Hata *et al.* 2005, Matthew *et al.* 2006, Manfredi *et al.* 2007, Arlot-Bonnemains *et al.* 2008, Mountzios *et al.* 2008). TGCTs are characterized, as other types of solid tumors, by aneuploidy, and show an altered expression pattern of the aurora kinases (Oosterhuis *et al.* 1989, Roelofs *et al.* 2000, Chieffi *et al.* 2004, Korkola *et al.* 2008, Baldini *et al.* 2010). This evidence identified the aurora kinases as new potential molecular targets also for the treatment of TGCT, especially against the most aggressive forms resistant to the conventional cisplatin-based chemotherapy (Nichols *et al.* 1994, Horwich *et al.* 2006, Fenner *et al.* 2008).

In the present study, we evaluated the *in vitro* effects of the aurora kinases functional inhibition on the TGCT-derived cell line NT2-D1, which carries the isochromosome 12p as diagnostic marker of the TGCT (Dmitrovsky *et al.* 1990). We demonstrated that treatment of these cells with aurora kinase MK-0457 inhibitor arrests proliferation in a time- and dose-dependent manner with an IC₅₀ of 17 nM, similar to the IC₅₀ values reported for this inhibitor on other cancer cell types and to the IC₅₀ reported to inhibit aurora kinase activity *in vitro*, the latter being 0.46 nM for Aurora-A, 18 nM for Aurora-B, and 4.6 nM for Aurora-C (Harrington *et al.* 2004). In addition, even the highest dose of MK-0457 used in the present study (200 nM) is well below the maximal plasma concentration recorded in phase I clinical study. In particular, in a dose-escalation phase I clinical study, in which the administration of the MK-0457 was performed by continuous 5-day i.v. infusion every 28 days, the drug was well tolerated by heavily pretreated patients and the dose limiting toxicity, consisting of asymptomatic neutropenia for more than 5 days,

Figure 4 Effects of the MK-0457 on aurora kinases protein levels and subcellular localization, centrosome maturation, TACC3 localization, and histone H3 phosphorylation in NT2-D1 cells. NT2-D1 cells were incubated for 6 h with 200 nM MK-0457 or DMSO as control. Following treatment, cells have been used for the subsequent experiments. (A) Western blot analysis of aurora kinases protein levels. (B) Subcellular localization of Aurora-A. Cells were fixed and stained for Aurora-A and pericentrin. (C) Subcellular localization of Aurora-B and phosphorylated histone H3. Cells were fixed and stained for Aurora-B and P-Histone H3. (D) Subcellular localization of Aurora-C in control NT2-D1 cells. Cells were fixed and stained for Aurora-C and β -tubulin. Scale bar, 10 μ m.

Figure 5 Effects of the MK-0457 on NT2-D1 cell colony formation in soft agar. The NT2-D1 cells were plated in soft agar onto 3.5 cm Petri dishes in the absence or in the presence of MK-0457 (200 nM). Treated and nontreated plates were photographed after plating to exclude the presence of cell aggregates (data not shown) and after 14 days of incubation. The colony size was determined using the MetaVue software, and those larger than 50 μm were scored. Photographs reported in the figure are representative of one out of three similar experiments.

was observed at 12 mg/m^2 per h. At the dose of 8 mg/m^2 per h, the maximal plasma concentration recorded was 650 nM, similar to that causing regressions in xenografts and well above the dose used in the present study (Harrington *et al.* 2004, Rubin *et al.* 2006, Mountzios *et al.* 2008).

Aurora-A kinase activity is required for the phosphorylation and localization of different proteins in the pericentrosomal area, involved in the mitotic spindle assembly (Carmena & Earnshaw 2004, Vagnarelli & Earnshaw 2004, Mori *et al.* 2007, Ulisse *et al.* 2007, Arlot-Bonnemains *et al.* 2008). Accordingly, we found that the treatment of NT2-D1 cells with MK-0457 causes major alterations in centrosome functions with abnormal spindle formation characterized by the presence of short microtubules. Histone H3 is a well-recognized target of Aurora-B kinase, and its phosphorylation is thought to mediate chromosome condensation during prophase (Crosio *et al.* 2002). In agreement with previous studies (Harrington *et al.* 2004, Arlot-Bonnemains *et al.* 2008), we showed, by means of immunofluorescence experiments, that the administration of MK-0457 to NT2-D1 cells completely abolished histone H3 phosphorylation.

Inhibition of aurora kinases activity impairs the completion of the mitotic phase, but the subsequent events are strictly conditioned by the integrity of the p53-p21^{WAF1/Cip1}-dependent post-mitotic checkpoint (Gizatullin *et al.* 2006). In cells having a normal p53-p21^{WAF1/Cip1} pathway, MK-0457 treatment arrests the cell cycle in a pseudo G₁ phase, characterized by 4N DNA content, and limited apoptosis. On the contrary, cells with compromised p53 function and/or insufficient or delayed p21^{WAF1/Cip1} induction undergo endoreduplication and apoptosis (Gizatullin *et al.* 2006). NT2-D1 cells have a wild-type p53 gene (Olivier *et al.* 2002) and, in agreement with the above findings, they stop in the tetraploid status following the exposure to MK-0457. After that, however, the NT2-D1 cells rapidly activate the apoptotic process, as demonstrated by cytofluorimetric analysis, time-lapse experiments, and caspase-3 activation.

In this context, it is worth to note that following activation p21^{WAF1/Cip1} may assume both pro- or anti-apoptotic functions in response to antitumor agents depending on cell types and cellular context (Liu *et al.* 2003).

In conclusion, we demonstrated that MK-0457 inhibits cell proliferation and induces apoptosis in the NT2-D1 cells. In addition, MK-0457 prevented the capability of the NT2-D1 cells from developing colonies in soft agar. These findings warrant further investigation to exploit the potential therapeutic value of aurora kinases inhibition in the treatment of TGCT, especially in patients whose type of cancer does not respond to the currently available therapeutic strategies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Andrews PW, Damjanov I, Simon D, Banting GS, Carlin C, Dracopoli NC & Fogh J 1984 Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation *in vivo* and *in vitro*. *Laboratory Investigation* **50** 147–162.
- Arlot-Bonnemains Y, Baldini E, Martin B, Delcros JG, Toller M, Curcio F, Ambesi-Impiombato FS, D'Armiento M & Ulisse S 2008 Effects of the Aurora kinase inhibitor VX-680 on anaplastic thyroid cancer-derived cell lines. *Endocrine-Related Cancer* **15** 559–568.
- Baldini E, Arlot-Bonnemains Y, Mottolese M, Sentinelli S, Antoniani B, Sorrenti S, Salducci M, Comini E, Ulisse S & D'Armiento M 2010 Deregulation of Aurora kinases genes expression in human testicular germ cell tumors. *Andrologia*. In press.
- Bray F, Richiardi L, Ekblom A, Pukkala E, Cuninkova M & Møller H 2006 Trends in testicular cancer incidence and mortality in 22 european countries: continuing increase in incidence and declines in mortality. *International Journal of Cancer* **118** 3099–3111.
- Carmena M & Earnshaw WC 2004 The cellular geography of Aurora kinases. *Nature Reviews. Molecular Cell Biology* **4** 842–854.
- Carvajal RD, Tse A & Schwartz GK 2006 Aurora kinases: new targets for cancer therapy. *Clinical Cancer Research* **12** 6869–6875.
- Chang D, Chen F, Zhang F, McKay BC & Ljungman M 1999 Dose-dependent effects of DNA-damaging agents on p53-mediated cell cycle arrest. *Cell Growth and Differentiation* **10** 155–162.
- Chieffi P, Troncone G, Caleo A, Libertini S, Linardopoulos S, Tramontano D & Portella G 2004 Aurora B expression in normal testis and seminomas. *Journal of Endocrinology* **181** 263–270.
- Crosio C, Fimia GM, Loury R, Kimura M, Okano Y, Zhou H, Sen S, Allis CD & Sassone-Corsi P 2002 Mitotic phosphorylation of histone H3: spatio-temporal regulation by mammalian Aurora kinases. *Molecular and Cellular Biology* **22** 874–885.
- Dmitrovsky E, Murty VV, Moy D, Miller WH Jr, Nanus D, Albino AP, Samaniego F, Bosl G & Chaganti RS 1990 Isochromosome 12p in non-seminoma cell lines: karyologic amplification of c-k-ras2 without point-mitotational activation. *Oncogene* **5** 543–548.
- von Eyben FE, Jacobsen GK & Skotheim RI 2005 Microinvasive germ cell tumor of the testis. *Virchows Archiv* **447** 610–625.

- Fenner MH, Beutel G & Grünwald V 2008 Targeted therapies for patients with germ cell tumors. *Expert Opinion on Investigational Drugs* **17** 511–522.
- Gizatullin F, Yao Y, Kung V, Harding MW, Loda M & Shapiro GI 2006 The Aurora kinase inhibitor VX-680 induces endoreduplication and apoptosis preferentially in cells with compromised p53-dependent postmitotic checkpoint function. *Cancer Research* **66** 7668–7677.
- Hanahan D & Weinberg RA 2000 The hallmarks of cancer. *Cell* **100** 57–70.
- Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T, Graham JA, Demur C, Hercend T, Diu-Hercend A *et al.* 2004 VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth *in vivo*. *Nature Medicine* **10** 262–267.
- Hata T, Furukawa T, Sunamura M, Egawa S, Motoi F, Ohmura N, Marumoto T, Saya H & Horii A 2005 RNA interference targeting Aurora kinase a suppresses tumor growth and enhances the taxane chemosensitivity in human pancreatic cancer cells. *Cancer Research* **65** 2899–2905.
- Horwich A, Shipley J & Huddart R 2006 Testicular germ-cell cancer. *Lancet* **367** 754–765.
- Katayama H, Ota T, Jisaki F, Ueda Y, Tanaka T, Odashima S, Suzuki F, Terada Y & Tatsuka M 1999 Mitotic kinase expression and colorectal cancer progression. *Journal of the National Cancer Institute* **91** 1160–1162.
- Katayama H, Sasaki K, Kawai H, Yuan ZM, Bondaruk J, Suzuki F, Fujii S, Arlinghaus RB, Czerniak BA & Sen S 2004 Phosphorylation by Aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53. *Nature Genetics* **36** 55–62.
- Korkola JE, Heck S, Olshen AB, Reuter VE, Bosl GJ, Houldsworth J & Chaganti RS 2008 *In vivo* differentiation of genomic evolution in adult male germ cell tumors. *Genes, Chromosomes & Cancer* **47** 43–55.
- Liu S, Bishop RW & Liu M 2003 Differential effects of cell cycle regulatory protein p21^{WAF/Cip1} on apoptosis and sensitivity to cancer therapy. *Drug Resistance Updates* **6** 183–195.
- Liu Q, Kaneko S, Yang L, Feldman RI, Nicosia SV, Chen J & Cheng JQ 2004 Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215. *Journal of Biological Chemistry* **279** 52175–52182.
- Looijenga LH & Oosterhuis JW 1999 Pathogenesis of testicular germ cell tumors. *Reviews of Reproduction* **4** 90–100.
- Lutke Holzik MF, Rapley EA, Koekstra HJ, Sleijfer DT, Nolte IM & Sijmons RH 2004 Genetic predisposition to testicular germ-cell tumor. *Lancet Oncology* **5** 363–371.
- Manfredi MG, Ecsedy JA, Meetze KA, Balani SK, Burenkova O, Chen W, Galvin KM, Hoar KM, Huck JJ, LeRoy PJ *et al.* 2007 Antitumor activity of MLN8054, an orally active small-molecule inhibitor of Aurora A kinase. *PNAS* **104** 4106–4111.
- Matthew N, Visintin C, Hartzoulakis B, Jarvis A & Selwood DL 2006 Aurora A and B kinases as targets for cancer: will they be selective for tumors? *Expert Review of Anticancer Therapy* **6** 109–120.
- Mitelman F 2000 Recurrent chromosome aberrations in cancer. *Mutation Research* **462** 247–253.
- Miyoshi Y, Iwao K, Egawa C & Noguchi S 2001 Association of centrosomal kinase STK15/BTAK mRNA expression with chromosomal instability in human breast cancers. *International Journal of Cancer* **92** 370–373.
- Mori D, Yano Y, Toyo-oka K, Yoshida N, Yamada M, Muramatsu M, Zhang D, Saya H, Toyoshima YY, Kinoshita K *et al.* 2007 NDEL1 phosphorylation by Aurora-A kinase is essential for centrosomal maturation, separation and TACC3 recruitment. *Molecular and Cellular Biology* **27** 352–367.
- Mountzios G, Terpos E & Dimopoulos M-T 2008 Aurora kinases as targets for cancer therapy. *Cancer Treatment Reviews* **34** 175–182.
- Nichols CR, Roth BJ, Loehrer PJ, Williams SD & Einhorn LH 1994 Salvage chemotherapy for recurrent germ cell cancer. *Seminars in Oncology* **21** 102–108.
- Nigg EA 2001 Mitotic kinases as regulators of cell division and its checkpoints. *Nature Reviews. Molecular Cell Biology* **2** 21–32.
- Noel EE, Perry J, Chaplin T, Mao X, Cazier JB, Joel SP, Oliver RTD, Young BD & Lu YJ 2008 Identification of genomic changes associated with cisplatin resistance in testicular germ cell tumor cell lines. *Genes, Chromosomes and Cancer* **47** 604–613.
- Olivier M, Eeles R, Hollstein M, Khan MA, Harris CC & Hainaut P 2002 The IARC TP53 Database: new online mutation analysis and recommendations to users. *Human Mutation* **19** 607–614.
- Oosterhuis JW & Looijenga LHJ 2005 Testicular germ cell tumours in a broader perspective. *Nature Reviews. Cancer* **5** 210–222.
- Oosterhuis JW, Castedo SM, de Jong B, Cornelisse CJ, Dam A, Sleijfer DT & Schraffordt Koops H 1989 Ploidy of primary germ cell tumors of the testis. Pathogenetic and clinical relevance. *Laboratory Investigation* **60** 14–21.
- Ota T, Suto S, Katayama H, Han ZB, Suzuki F, Maeda M, Tanino M, Terada Y & Tatsuka M 2002 Increased mitotic phosphorylation of histone H3 attributable to AIM-1/Aurora-B overexpression contributes to chromosome number instability. *Cancer Research* **62** 5168–5177.
- Roelefs H, Mostert MC, Pompe K, Zafarana G, van Oorschot M, van Gurp RJ, Gillis AJ, Stoop H, Beverloo B, Oosterhuis JW *et al.* 2000 Restricted 12p amplification and RAS mutation in human germ cell tumors of the adult testis. *American Journal of Pathology* **157** 1156–1166.
- Rubin EH, Shaoiro GI, Stein MN, Watson P, Bergstrom D, Xiao A, Clark JB, Freedman SJ & Eder IP 2006 A phase I clinical and pharmacokinetic (PK) trial of the Aurora kinase (AK) inhibitor MK-0457 in cancer patients (abstract 3009). *Journal of Clinical Oncology* (June 20 Supplement) **24** 18S.
- Sakakura C, Hagiwara A, Yasuoka R, Fujita Y, Nakanishi M, Masuda K, Shimomura K, Nakamura Y, Inazawa J, Abe T *et al.* 2001 Tumour-amplified kinase BTAK is amplified and overexpressed in gastric cancers with possible involvement in aneuploid formation. *British Journal of Cancer* **84** 824–831.
- Sokoloff MH, Joyce GF & Wise M 2007 Urologic disease in american project. Testis cancer. *Journal of Urology* **177** 2030–2041.
- Takahashi T, Futamura N, Yoshimi N, Sano J, Katada M, Takagi Y, Kimura M, Yoshioka T, Okano Y & Saji S 2000 Centrosomal kinases, HsAIRK1 and HsAIRK3, are overexpressed in primary colorectal cancers. *Japanese Journal of Cancer Research* **91** 1007–1014.
- Tanner MM, Grenman S, Koul A, Johannsson O, Meltzer P, Pejovic T, Borg A & Isola JJ 2000 Frequent amplification of chromosomal region 20q12–q13 in ovarian cancer. *Clinical Cancer Research* **6** 1833–1839.
- Tatsuka M, Sato S, Kitajima S, Suto S, Kawai H, Miyauchi M, Ogawa I, Maeda M, Ota T & Takata T 2005 Overexpression of Aurora-A potentiates HRAS-mediated oncogenic transformation and is implicated in oral carcinogenesis. *Oncogene* **24** 1122–1127.
- Ulisse S, Delcros J-G, Baldini E, Toller M, Curcio F, Giacomelli L, Prigent C, Ambesi-Impimbato FS, D'Armiento M & Arlot-Bonnemains Y 2006 Expression of Aurora kinases in human thyroid carcinoma cell lines and tissues. *International Journal of Cancer* **119** 275–282.
- Ulisse S, Baldini E, Toller M, Delcros JG, Guého A, Curcio F, De Antoni E, Giacomelli L, Ambesi-Impimbato FS, Bocchini S *et al.* 2007 Transforming acid coiled-coil 3 and Aurora-A interact in human thyrocytes and their expression is deregulated in thyroid cancer tissues. *Endocrine-Related Cancer* **14** 827–837.
- Vagnarelli P & Earnshaw WC 2004 Chromosomal passengers: the four-dimensional regulation of mitotic events. *Chromosoma* **113** 211–222.
- Yan X, Cao L, Li Q, Wu Y, Zhang H, Saiyin H, Liu X, Zhang X, Shi Q & Yu L 2005 Aurora C is directly associated with Survivin and required for cytokinesis. *Genes Cells* **10** 617–626.
- Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A, Brinkley BR & Sen S 1998 Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. *Nature Genetics* **20** 189–193.

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