Inhibition of the aurora kinases suppresses \textit{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 \textit{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\textsubscript{50} = 17.2 ± 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. Cytosfluorimetric analysis confirmed that the treatment with MK-0457 for 6 h induced NT2-D1 cells accumulation in the G\textsubscript{2}/M phase of the cell cycle and the subsequent appearance of sub-G\textsubscript{0} 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 \textit{in vitro} growth and tumorigenicity of NT2-D1 cells, and indicate its potential therapeutic value for TGCT treatment.


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 above-mentioned agents. In particular, MK-0457 (VX-680), has been shown to inhibit cell 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 SuperSignal 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 dimethyl sulfoxide (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 reincubated 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 ± 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).
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 $\leq 2$ 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 immuno-fluorescence (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...
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 mm², with a mean area of 639 ± 129 μm², 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 μm.
was observed at 12 mg/m² per h. At the dose of 8 mg/m² 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, Moutzios 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-p21WAF1/Cip1-dependent post-mitotic checkpoint (Gizatullin et al. 2006). In cells having a normal p53-p21WAF1/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 p21WAF1/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 p21WAF1/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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