Effects of selective inhibitors of Aurora kinases on anaplastic thyroid carcinoma cell lines

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Abstract

Aurora kinases are serine/threonine kinases that play an essential role in cell division. Their aberrant expression and/or function induce severe mitotic abnormalities, resulting in either cell death or aneuploidy. Overexpression of Aurora kinases is often found in several malignancies, among which is anaplastic thyroid carcinoma (ATC). We have previously demonstrated the in vitro efficacy of Aurora kinase inhibitors in restraining cell growth and survival of different ATC cell lines. In this study, we sought to establish which Aurora might represent the preferential drug target for ATC. To this end, the effects of two selective inhibitors of Aurora-A (MLN8237) and Aurora-B (AZD1152) on four human ATC cell lines (CAL-62, BHT-101, 8305C, and 8505C) were analysed. Both inhibitors reduced cell proliferation in a time- and dose-dependent manner, with IC50 ranges of 44.3–134.2 nM for MLN8237 and of 9.2–461.3 nM for AZD1152. Immunofluorescence experiments and time-lapse videomicroscopy yielded evidence that each inhibitor induced distinct mitotic phenotypes, but both of them prevented the completion of cytokinesis. As a result, polyploidy increased in all AZD1152-treated cells, and in two out of four cell lines treated with MLN8237. Apoptosis was induced in all the cells by MLN8237, and in BHT-101, 8305C, and 8505C by AZD1152, while CAL-62 exposed to AZD1152 died through necrosis after multiple rounds of endoreplication. Both inhibitors were capable of blocking anchorage-independent cell growth. In conclusion, we demonstrated that either Aurora-A or Aurora-B might represent therapeutic targets for the ATC treatment, but inhibition of Aurora-A appears more effective for suppressing ATC cell proliferation and for inducing the apoptotic pathway.

Key Words

- anaplastic thyroid cancer
- Aurora kinases
- Aurora inhibitors
- MLN8237
- AZD1152
- therapy
- cell cycle

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Introduction

Anaplastic thyroid carcinoma (ATC) is a rare, highly aggressive form of thyroid cancer and overall one of the most deadly cancers, unresponsive to any available radio- and chemotherapy (Smallridge et al. 2012). On presentation, patients with ATC frequently display invasion of adjacent tissues and distant metastases, and their median survival is about 5 months. Although derived from follicular epithelium of the thyroid gland, ATC cells do not retain any biological feature of the original cell type as a result of multiple mutational events leading to dedifferentiation. A number of gene expression studies in ATC yielded evidence of the upregulation of genes involved in cell cycle progression and chromosome segregation, among which were the Aurora kinases (Salvatore et al. 2007, Wiseman et al. 2007, Rusinek et al. 2011). In particular, Aurora-A has been identified among the most frequently and strongly overexpressed proteins in ATC, and high expression of Aurora-B has been also reported (Sorrentino et al. 2005, Ulisse et al. 2006, 2007, Wiseman et al. 2007).

The Aurora kinases are a family of serine/threonine kinases essential for the mitotic process and involved in all steps from prophase to anaphase (Baldini et al. 2012a). Aurora-A localizes to centrosomes and spindle poles, and drives centrosome maturation and separation, timing of mitotic entry, bipolar spindle assembly, alignment of chromosomes on the metaphasic plate, cytokinesis, and return to G1 (Nikonova et al. 2013). Aurora-B, together with the proteins INCENP, Survivin and Borealin, is a component of the chromosomal passenger complex (CPC), which localizes along the chromosome arms in prophase, concentrates in the inner centromere region from prometaphase to metaphase, moves to the central spindle and cortex in anaphase, and remains in the midbody in telophase (Lens et al. 2010). The CPC ensures accurate chromosome segregation by regulating chromosome structure, cohesin removal from the chromosomal arms, spindle formation, kinetochore assembly, correction of non-bipolar chromosome-microtubule connections, spindle assembly checkpoint (SAC), and cytokinesis (van der Waal et al. 2012). Aurora-C is highly similar to Aurora-B and joins the CPC, but it has been found expressed substantially only in germ cells during spermatogenesis and oogenesis, included meiosis, and in some cancer cell lines (Gabillard et al. 2011, Baldini et al. 2012a,b).

Aberrant expression and/or function of one or more Aurora kinases result in abnormal cell divisions with consequent generation of aneuploid cells. Genetic amplification and overexpression of Aurora-A are often observed in solid tumors, and correlate positively with aneuploidy, supernumerary centrosomes, defective mitotic spindles, and resistance to apoptosis (Baldini et al. 2012a,b, Kollareddy et al. 2012). Similarly, although not arising from gene amplification or mutations, Aurora-B overexpression has been described in several cancer types, where it is associated with genomic instability and aneuploidy. Consistently with the phenotype expected for deregulation of these proteins, ATCs are typically aneuploid and exhibit a high degree of numerical and structural chromosomal abnormalities (Smallridge et al. 2012). Thus, Aurora kinases have been considered as potential molecular targets for anticancer agents in ATC.

In recent years, our group has analyzed the effects of some small molecule pan-inhibitors of Aurora kinases, including VX-680/MK-0457 (Arlot-Bonnemains et al. 2008), SNS-314 (Baldini et al. 2012b), and ZM447439 (Baldini et al. 2013), on human ATC-derived cell lines. Our findings indicated that all the inhibitors reduced considerably the growth and survival of the different ATC cells tested, which prompted us to further explore the anti-tumoral properties of this class of compounds. In this study, we compared the effects of two small-molecule selective inhibitors of Aurora-A and Aurora-B on proliferation, cell cycle, survival, and in vitro tumorigenicity of four ATC cell lines, in order to establish which Aurora kinase might represent the preferential drug target for ATC.

Materials and methods

Cell cultures

The ATC-derived cells CAL-62, 8305C, 8505C, and BHT-101 were purchased from the German Collection of Microorganisms and Cell Cultures (DMSZ, Braunschweig, Germany) and cultured in appropriate media as indicated by the supplier: CAL-62 in DMEM plus 10% fetal bovine serum (FBS), 8305C and 8505C in RPMI plus 10% FBS, and BHT-101 in DMEM plus 10% FBS and 0.5% human serum. All the media were supplemented with 2 mM glutamine. The unique DNA profile of each cell line was authenticated by the supplier using multiplex PCR of minisatellite markers. For all the experiments described below the different cell lines were used at passage numbers 3–6.

Aurora kinase inhibitors

MLN8237 (Alisertib) is an Aurora-A inhibitor with IC_{50} of 1.2 nM, having >200-fold higher selectivity for Aurora-A.
than Aurora-B (Manfredi et al. 2011). AZD1152 (Barasertib) is an Aurora-B inhibitor with an IC$_{50}$ of 0.37 nM, about 3000-fold more selective for Aurora-B over Aurora-A (Wilkinson et al. 2007). Both were acquired from Selleck Chemicals (Houston, TX, USA). The stock solutions 10 nM were prepared by diluting the powder in DMSO, aliquoted and stored at a $-20^\circ$C until use. In all the experiments, controls cultures were performed by administrating the vehicle (DMSO) only at a volume corresponding to the highest volume of inhibitor used for treated cells.

**Proliferation assay**

The dose- and time-dependent effects of the inhibitors on cell proliferation were evaluated by means of colorimetric assay and cell count. At first, cells were seeded in 96-multiwell culture plates and treated with different concentrations of MLN8237 (1–1000 nM), AZD1152 (1–2000 nM) or DMSO for 4 days, replacing media every 48 h. At the end of incubation, 5-bromo-2-deoxyuridine (BrdU) incorporation into cellular DNA was measured using a Cell Proliferation ELISA BrdU Kit (Roche Diagnostics). In addition, cells were seeded in six-well culture plates and subjected to the same treatments, after that they were detached by trypsin/EDTA, harvested in culture medium, and diluted 1:2 with Trypan blue, and then viable cells were counted in the Burker hemocytometer.

Next, analogous experiments were accomplished by treating cells for different periods of time (1–6 days) with MLN8237 250 nM, AZD1152 100 nM (8505C and CAL-62), AZD1152 1 µM (BHT-101 and 8305C), or vehicle alone (DMSO). These inhibitor concentrations, exerting maximal anti-proliferative effects, were employed in all the subsequent experiments.

**Western blotting analysis**

The cells were treated with each inhibitor or the vehicle alone for 24 h and then total cell protein extracts were prepared and western blotting analysis was carried out as described previously (Baldini et al. 2011). The following primary antibodies were employed: anti-phospho-histone H3 (Ser10) 1:2000 (#3377 Cell Signaling Technology, Danvers, MA, USA), anti-phospho-Aurora-A/B/C 1:1000 (#2914 Cell Signaling Technology), and anti-β-tubulin 1:10 000 (T4026 Sigma–Aldrich). After washing, the membranes were incubated with appropriate HRP-conjugated secondary antibodies 1:1000 (Thermo Fisher Scientific, Rockford, IL, USA) and developed using the LiteAblot EXTEND chemiluminescent substrate (Euroclone, Milan, Italy).

**Immunofluorescence**

Cells were cultured on glass coverslips and treated with AZD1152, MLN8237, or vehicle alone for 24 h, and then fixed in cold methanol for 10 min. The coverslips were rinsed with PBS, saturated with 3% BSA in PBS for 1 h, and incubated with antibodies, anti-phospho-Aurora-A/B/C 1:100, anti-phospho-histone H3 (Ser10) 1:1000, or anti-β-tubulin 1:2000, in PBS with 1.5% BSA for 2 h. After washing, the coverslips were incubated with TRITC- and FITC-conjugated anti-mouse/anti-rabbit antibodies (Jackson Laboratories, West Grove, PA, USA) 1:100 in PBS with 1.5% BSA for 1 h, washed in PBS, and then mounted in Vectashield medium (Vector Laboratories, Burlingame, CA, USA) containing 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI). The cells were observed using a Leica-DMRXA microscope and photographed with a digital camera.

**Time-lapse analysis**

Cells at about 50% confluence in a culture dish were put in fresh complete medium containing AZD1152, MLN8237, or vehicle alone, and placed under a Leica DM-IRBE microscope equipped with an incubation chamber at 37 $^\circ$C and 5% CO$_2$. Cell pictures were acquired every 5 min for 24 h using a microscope photo camera.

**Flow cytometric analysis**

Cells were treated for 48 h with AZD1152, MLN8237, or vehicle alone, and then collected in PBS by scraping, centrifuged at 180 g for 5 min, and fixed in 70% ice-cold ethanol for 1 h. After washing with PBS, cells were incubated with 0.5 mg/ml RNAse at +37 $^\circ$C for 1 h, labeled with 10 µg/ml propidium iodide, and analyzed on FACS using the FACScalibur Flow cytometer and CellQUEST Software (BD Biosciences, San Jose, CA, USA).

**Apoptosis assay**

The induction of apoptosis following cell exposure to the inhibitors was evaluated by means of the Cell Death Detection ELISA PLUS (Roche Diagnostics). The cells were seeded in 96-well culture plates with AZD1152, MLN8237, or vehicle alone, and incubated for 48, 72, 96, or 120 h. At the end of the incubation time, the supernatant was removed from each well and stored at $+4^\circ$C. A 200 µl volume of lysis buffer per well was added and samples were processed according to the manufacturer’s instructions. The ELISA was performed with 20 µl lysis buffer.
(indicative of apoptosis) or supernatant (indicative of necrosis) in triplicate.

Analysis of pro-apoptotic and anti-apoptotic proteins

The induction of apoptosis was further investigated by means of the Bio-Plex Pro RBM apoptosis assays performed using three different kits (Bio-Rad), each analyzing specific panels of apoptotic proteins. On the basis of the results from the time-course of DNA fragmentation, cells were treated with AZD1152, MLN8237, or vehicle alone for 48 h (BHT-101), and 72 h (8305C and 8505C), and CAL-62 with AZD1152 for 120 h. Then, cells were trypsinized, collected, and processed following the manufacturer’s instructions. Both cytosolic and nuclear + mitochondrial fractions were prepared and subjected to analysis in triplicate.

Colonial formation in soft agar

Petri dishes containing soft agar cell cultures supplemented with inhibitors or vehicle alone were prepared as described previously (Baldini et al. 2012b). After 2 weeks of incubation, the dishes were photographed. Nine photos for each treatment were analyzed by means of the ImageJ program, scoring colonies with diameter ≥ 50 μm.

Statistical analysis

All the results expressed as mean ± S.E.M. were obtained from three independent experiments. The IC_{50} were calculated from the dose–response curves by curve fitting with four-parameter logistic functions using the ALLFIT program (DeLean et al. 1978). The statistical significance of data was evaluated by the Wilcoxon’s rank-sum test, with a P value threshold of 0.05.

Results

Dose- and time-dependent effects of MLN8237 and AZD1152 on the proliferation of ATC-derived cells

The Aurora inhibitors were administered to the cell cultures at different doses and times of incubation. As showed in Fig. 1, both inhibitors were able to reduce the ATC cell number up to approximately ~ 95% in a dose-dependent manner. At fully effective doses, the proliferation decreased significantly after only 24 h of treatment, and reached the minimum rate within 96 h (Fig. 2).

Selectivity of the Aurora inhibitors at effective anti-proliferative doses

In order to ascertain that the inhibitors maintained their selectivity for Aurora-A and Aurora-B at doses exerting maximal anti-proliferative effects, western blotting experiments were carried out. The inhibition of either Aurora-A or Aurora-B activity was determined by means of an antibody, which detects endogenous levels of Aurora-A/B/C only when phosphorylated at their autophosphorylation sites, i.e. Thr288, Thr232, or Thr198 respectively. The Aurora-B activity was also evaluated by assessing the phosphorylation of histone H3 on Ser10, specifically due to this kinase. From Fig. 3 it is evident that the treatment with MLN8237 abolished the autophosphorylation of Aurora-A but not that of Aurora-B and Aurora-C, and not even the phosphorylation of H3(Ser10). In contrast, treatment with AZD1152 abrogated the autophosphorylation of Aurora-B and Aurora-C as well as the phosphorylation of H3(Ser10), without affecting the autophosphorylation of Aurora-A. The protein levels of the three Aurora kinases did not vary in the presence of the two inhibitors, as expected for functional inhibitors (results not shown).
Effects of MLN8237 and AZD1152 on the mitosis of ATC cells

Immunofluorescence experiments were carried out to observe the mitotic features of ATC cells lacking either Aurora-A or Aurora-B activity. Both inhibitors induced alterations in the spindle morphology in all the cell lines (Fig. 4 and Supplementary Figs 1, 2 and 3, see section on supplementary data given at the end of this article). In particular, cells treated with either AZD1152 or MLN8237 showed chromosomal misalignment on the metaphasic plate, but MLN8237 caused also centrosome amplification and disruption of spindle organization. The selective inhibition of the Aurora kinases was confirmed using the anti-phospho-Aurora-A/B/C antibody described earlier, which detected only phospho-Aurora-A in the spindle poles of cells treated with AZD1152 and only chromosome-associated phospho-Aurora-B in cells treated with MLN8237 (Fig. 4). In addition, the phosphorylation of H3(Ser10) was abolished only by AZD1152. No anaphase or cytokinesis could be detected in the presence of inhibitors.

Effects of MLN8237 and AZD1152 on cycle progression and ploidy of ATC cells

The cell cycle of ATC cells exposed to each inhibitor was observed by time-lapse videomicroscopy. Control cells accomplished mitosis and cytokinesis, while cells treated with both AZD1152 and MLN8237 underwent mitotic slippage, which is escape from mitosis without chromosome segregation and cell division (Fig. 5 and Supplementary Fig. 4, see section on supplementary data given at the end of this article). In MLN8237-treated cells, a provisional arrest in G2/M phase was also observed. Consistent with these findings, remarkable increases in ploidy after treatment with MLN8237 (Fig. 4).
Figure 4
Immunofluorescence (IF) of BHT-101 cells treated with MLN8237 or AZD1152. BHT-101 cells were exposed to MLN8237 (250 nM), AZD1152 (1 μM), or DMSO (control) for 24 h. Ph-Aur-A/B/C, auto-phosphorylated forms of Aurora-A/B/C; Ph-H3, histone H3 phosphorylated on Ser10.
AZD1152 and accumulation of cells with DNA=4N (G2/M/tetraploid) and DNA >4N (polyploid) after treatment with MLN8237 (Fig. 6) were revealed in the cell lines CAL-62, 8305C, and 8505C by the cytofluorometric analysis at 48 h incubation. At the same time, a fraction of cells with DNA <2N, likely corresponding to apoptotic ones, appeared in all the treated cultures.

Effects of MLN8237 and AZD1152 on ATC cell apoptosis

The induction of apoptosis was examined more thoroughly at different times of incubation by measuring the intracellular DNA fragmentation in a quantitative sandwich-enzyme immunoassay. In the same experiments, necrosis was also estimated from the DNA content of cell supernatants. MLN8237 caused a significant increase in apoptosis in all the ATC cells after 48 h treatment (data not shown), which became more marked at 72 h in three out of four cell lines (CAL-62, 8305C, and 8505C) (Fig. 7A). At these times, AZD1152 produced similar results in all the cells except CAL-62 (Fig. 7A). Necrosis was observed to take place concurrently to apoptosis in CAL-62, 8305C, and 8505C cells exposed to MLN8237, and in 8305C exposed to AZD1152 (Fig. 7B). Incubation with AZD1152 was prolonged up to 96 and 120 h for CAL-62. These cells were capable of proceeding with endoreduplications longer than the other ATC lines, accumulating a very high content of DNA and cytoplasm before they died. After 120 h treatment, necrosis without apoptosis was finally observed (Fig. 7C).

Effects of MLN8237 and AZD1152 on pro-apoptotic and anti-apoptotic proteins in the ATC cell lines

Cell death was further investigated by determining the levels of several proteins involved in the apoptotic pathway in control and treated cells (Figs 8 and 9). Specifically, we quantified BAX, BAK, BAD, BIM, BCL-XL, MCL1, and the complexes BAK/BCL-XL, BAK/MCL1, and BAX/BCL2 in the mitochondrial membranes, together with survivin, SMAC/DIABLO and active caspase 3 in the cytosol. Upon apoptotic stimuli, BAK and BAX form...
oligomer channels in the mitochondrial membrane allowing release of cytochrome c. AZD1152 and MLN8237 increased BAK in 8305C, as did MLN8237 in BHT-101, while both treatments reduced BAK in CAL-62 (Fig. 8). Both inhibitors induced BAX in all the cells, except for MLN8237 in CAL-62 (Fig. 8). BCL-XL and MCL1 heterodimerize with BAK and BAX preventing their oligomerization. Both inhibitors increased BCL-XL, but only in CAL-62 and 8305C; interestingly, the complex of BCL-XL with BAK, its main target, does not always reflect the BCL-XL variation profile (Fig. 9). MCL1 increased in 8305C treated with both inhibitors, and in BHT-101 treated with MLN8237; the complex of MCL-1 with BAK, its main target, showed a similar trend (Fig. 9). Phosphorylated BAD is bound to protein 14-3-3 in the cytosol of healthy cells and, when apoptosis is triggered, it is dephosphorylated and released, and then moves on mitochondrial membrane where it inhibits the activity of BCL2 family proteins. AZD1152 and MLN8237 increased the mitochondria-bound BAD in CAL-62 and 8305C cells (Fig. 8). Another feature of apoptotic cells is the export of SMAC/DIABLO from mitochondria into the cytosol where it reverses IAP (inhibitor of apoptosis family) inhibition of caspase 9. Both inhibitors reduced cytoplasmic SMAC levels in CAL-62 and 8305C cells. Finally, the inhibitors increased active caspase 3, a primary regulator of apoptosis-associated proteolysis, in all ATC cells (Fig. 8).
Lastly, we evaluated the ability of each inhibitor to impair the growth of cell colonies in soft agar. After three weeks of incubation, both inhibitors displayed the same anti-proliferative efficacy observed in adherent cultures, impairing considerably or totally the colony formation of all the cell lines analyzed (Fig. 10).

Discussion

In the past decade, the kinases Aurora-A and Aurora-B, primarily active during mitosis and essential for its proper completion, have become the subject of increasing interest as potential targets for anticancer therapy. Preclinical studies and early phase I and II clinical trials carried out with several Aurora inhibitors have provided encouraging results for various cancer types. Recently, our group has demonstrated the ability of different small-molecule pan-inhibitors of Auroras to halt proliferation and to induce apoptosis of ATC-derived cells (Arlot-Bonnemains et al. 2008, Baldini et al. 2012a, 2013). In the present work, we sought to verify whether analogous effects are achievable by using selective Aurora inhibitors, and which kinase might represent the preferential drug target for ATC.

Our findings indicate that both MLN8237, targeting Aurora-A, and AZD1152, targeting Aurora-B, are highly effective in reducing ATC cell growth dose- and time-dependently, but MLN8237 displayed a lower IC₅₀ range. To the best of our knowledge, only one paper has been published reporting the effects of AZD1152 in three of the ATC cell lines evaluated in this study, i.e. CAL62, BHT-101, and 8505C (Libertini et al. 2011), and no reports are available on the effects of MLN8237 on ATC cells. The IC₅₀ obtained by Libertini and colleagues for CAL-62 and 8505C are similar to those calculated by us, while the IC₅₀ of BHT-101 is significantly lower. However, we noticed that these authors named the latter cell line BHT101-5 without any specification. It has to be considered that BHT-101 cells were originally described as a heterogeneous population containing epithelial-like and fibroblast-like cells; the epithelial-like clone 1 and the fibroblast-like clone 3, isolated from the parental line, differed in drug sensitivity (Pályi et al. 1993). Therefore, it can be supposed that the discrepancy in IC₅₀ derives from differences between the cells clones analyzed. It is worth noting that the IC₅₀ obtained for the different cell lines with each inhibitor differs depending on which proliferation test was used, cell count, or BrdU incorporation (see Table 1). Specifically, for CAL-62 and 8505C cells treated with AZD1152, the IC₅₀ calculated from cell count are considerably lower than IC₅₀ calculated from BrdU incorporation. These results can be explained by considering that DNA synthesis is no longer proportional to cell number if cells undergo abortive divisions. At 4 days of incubation, the more cells have been capable of carrying...
out multiple round of cell cycle without cytokinesis the more BrdU incorporation underestimates the anti-proliferative effect of AZD1152. Indeed, the discrepancy between the two IC_{50} is maximal for CAL-62 exposed to AZD1152, which continue to replicate for 4 days, becoming giant cells until they die. In contrast, the IC_{50} resulting from cell counts are higher than the IC_{50} resulting from the BrdU assay in BHT-101 and 8305C treated with AZD1152 (100 nM) or MLN8237 (250 nM) for 72 h; and 8505C with AZD1152 (100 nM) or MLN8237 (250 nM) for 72 h. Then, protein extracts were prepared, separating the cytosolic fraction from the nuclear + mitochondrial fraction, and proteins were detected with the Bio-Plex Pro RBM apoptosis assays. The values for treated cells were normalized against those for control cells.

Thus, inactivation of Aurora-A might alter the amount of geminin in the S phase, affecting proper DNA replication. Otherwise, the quota of DNA-synthesizing cells in the culture might be reduced owing to the temporary block in mitosis occurring in Aurora-A depleted cells.

In western blotting experiments, MLN8237 was shown to abrogate only Aurora-A activity, while AZD1152 appeared capable of inhibiting Aurora-C as well as Aurora-B. This result is not unexpected, considering that these proteins have 68.7% identity and, to our knowledge, there are no commercially available inhibitors that are claimed to be restricted to either
Aurora-B or Aurora-C. On the other hand, given that Aurora-B and Aurora-C participate in the same CPC complex and are thought to play similar roles in mitosis (Fernández-Miranda et al. 2011), their simultaneous inhibition has the advantage of preventing possible functional compensations.

Inactivation of Aurora-A has been reported to induce the formation of either monopolar or multipolar spindles with microtubules of decreased mass and aberrant morphology, unable to align the condensed chromosomes on the metaphasic plate correctly. As a consequence, the SAC turns on to block the cells in G2/M for a time, until it is overridden through mitotic slippage and tetraploidization or induction of apoptosis, depending on the cell type. The escape from mitosis in the presence of an unsatisfied SAC has been shown to require the proteasome-dependent

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**Figure 10**

Anchorage-independent growth of ATC cells treated with MLN8237 or AZD1152. Cells were plated in soft agar medium containing MLN8237 (250 nM), AZD1152 (100 nM) (8505C and CAL-62), AZD1152 (1 μM) (BHT-101 and 8305C) or DMSO (control), and incubated for 14 days. Nine photos for each treatment and control were acquired (A), and colonies ≥ 50 μm diameter were counted (B). *P < 0.05.
degradation of cyclin B, with consequent inhibition of the cyclin B/CDK1 activity (Brito & Rieder 2006). Inactivation of Aurora-B causes impairment of SAC, onset of premature anaphase with unaligned and lagging chromosomes, abrogation of cleavage furrow ingression, and of the abscission checkpoint from anaphase to cytokinesis, leading to cytokinesis failure and tetraploidy (Carmena & Earnshaw 2003, Lapenna & Giordano 2009).

Depending on the inhibitor employed, ATC cells showed mitotic abnormalities consistent with the inhibition of Aurora-A or Aurora-B. However, mitotic slippage and generation of polyploid cells were common effects of both inhibitors, presumably due to the lack of functional p53 in all the cell lines tested in this study, which allowed them to escape the post-mitotic checkpoint, to reenter the cell cycle and to endoreplicate (Gioanni et al. 1991, Olivier et al. 2002). Interestingly, the inhibitors triggered diverse cell fates with variable times of response in the different cell lines. In particular, BHT-101 exposed to either MLN8237 or AZD1152 exhibited just a little increase in ploidy and reached the maximum apoptotic index in 48 h without necrosis. BHT-101 has a hypertetraploid karyotype and a doubling time of 24 h, it is reasonable to assume that they do not tolerate a further increase in ploidy and trigger apoptosis soon after the first abortive division. In contrast, CAL-62 were committed to apoptosis by MLN8237, but not by AZD1152, in presence of which they developed a very high degree of polyplody, enduring for 5 days of treatment before dying by necrosis. At 72 h incubation both apoptosis and necrosis were strongly induced in 8305C by the inhibitors, while in 8505C apoptosis prevailed over necrosis with MLN8237, and necrosis was absent with AZD1152. The concomitant appearance of two pathways of cell death is probably attributable to an autolytic necrotic outcome of apoptotic cells, i.e. secondary necrosis (Silva et al. 2008).

Recently, evidence has been presented that delaying mitotic slippage provides more time for accumulation of death signals, which might account for the better efficacy of selective Aurora-A inhibition in inducing apoptosis (Gascoigne & Taylor 2008). How prolonged mitotic arrest activates the cell death machinery is not known. It has been proposed that during this period, the cells acquire telomeric DNA damage foci responsible for a p53-independent caspase activation after the cells exit mitosis and progress into G1 phase (Hayashi & Karlsceder 2013). The death of multinucleated ATC cells produced by the Aurora-B inhibitor might be a consequence of the so-called ‘aneuploidy-associated stresses’, including genetic instability, increased energy needs, and proteotoxic effects (Tang & Amon 2013). The final cell death modality presumably depends on differences in the expression profiles of proteins involved in cell cycle regulation and apoptosis inception between the various cell lines.

In this regard, we found that in different ATC cell lines exposed to the same inhibitor, the levels of apoptotic proteins vary with distinct profiles. Nonetheless, a common feature of all cells is that both inhibitors induce the augmentation of at least one of the pore-forming proteins BAK and BAX on the mitochondrial membranes. Assembly of these proteins is impaired by binding to anti-apoptotic proteins (e.g. BCL-XL, BCL2, and MCL1), which in turn are neutralized by pro-apoptotic BH3-only proteins (e.g. BAD and BIM). Although some of these proteins and/or their complexes were increased or diminished on the mitochondrial membranes depending on inhibitor and cell line, the upshot of the combined pro- and anti-apoptotic actions was always activation of caspase 3. Unexpectedly, CAL-62 treated with AZD1152 showed the most pronounced augmentation of active caspase 3 although DNA fragmentation was absent. However, evidence has been provided that cells committed to apoptosis are instead forced to die by necrosis when energy levels are rapidly compromised, as occurs in highly polyploid cells (Nicotera & Melino, 2004, Tang & Amon 2013).

### Table 1 Estimated IC50 for MLN8237 and AZD1152 in the different ATC-derived cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MLN8237</th>
<th>AZD1152</th>
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<tbody>
<tr>
<td>CAL-62</td>
<td>76.7 ± 12.4</td>
<td>9.2 ± 0.8</td>
</tr>
<tr>
<td>BHT-101</td>
<td>134.2 ± 7.5</td>
<td>461.3 ± 33.7</td>
</tr>
<tr>
<td>8305C</td>
<td>44.6 ± 2.7</td>
<td>287.8 ± 85.9</td>
</tr>
<tr>
<td>8505C</td>
<td>44.3 ± 5.9</td>
<td>342.1 ± 18.1</td>
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Cell proliferation was evaluated by means of cell count or BrdU incorporation as specified in the ‘Materials and methods’ section. The IC50 values were calculated with the ALLFIT Software (National Institutes of Health, Bethesda, MD, USA) using data from three independent experiments.
Anticancer drugs are all conceived to induce cancer-cell-selective death, and both MLN8237 and AZD1152 fulfill this task in ATC cells. Nonetheless, the different death pathways activated by AZD1152 might represent a discriminating factor for its clinical use. In vivo, apoptosing cells are usually phagocytosed by scavenger cells before the transition to secondary necrosis, whereas necrosing cells are engulfed less efficiently and in the form of fragments after cytoplasmic membrane damage and cell rupture (Silva et al. 2008). As a consequence, pro-inflammatory and immunostimulatory responses might take place following a massive cell necrosis, whose effects on malignant cells might be either positive (e.g. a tumor-promoting action of cytokines and macrophage-associated angiogenic and growth factors) or negative (e.g. initiation of an anti-tumor immune response). Whether the local inflammation provoked by necrosis would be beneficial or detrimental for tumor growth is still a matter of debate. Moreover, the immune-mediated injury to peritumoral tissues as well as the potential onset of autoimmune diseases might represent important therapeutic barriers.

Unlike ADZ1152, MLN8237 was able to activate apoptosis in all the ATC cells tested and, compared with AZD1152, displayed IC_{50} values closer to those that we previously obtained with the pan-inhibitors of Aurora kinases SNS-314 and VX-680 for the same cell lines by means of the same proliferation assay used in this study (Arlot-Bonnemains et al. 2008, Baldini et al. 2012a, b).

Pan-Aurora inhibitors are known to produce a cellular phenotype resembling the inactivation of Aurora-B rather than Aurora-A, which prompted some authors to consider Aurora-B to be the main target of this class of compounds. However, this is not a correct conclusion because the effects of Aurora-A inhibition are masked by those of Aurora-B inhibition at phenotypic level. Specifically, as described above, inhibition of Aurora-A but not of Aurora-B leads cells to stop in G2/M due to activation of the SAC; the latter being impaired in cells depleted of Aurora-B, G2/M arrest is not achievable with dual inhibitors. Moreover, polyploidization is often reported as a signature of Aurora-B inhibition, but it has been observed also in tumor cells following selective inhibition of Aurora-A (Wysong et al., 2009, Nair et al., 2012, Sehdev et al., 2012). Given that malignant cells are largely heterogeneous for gene mutations and chromosome abnormalities, they might respond to pan-Aurora inhibitors because they are more sensitive to Aurora-A inactivation, or to Aurora-B inactivation, or equally to both. Hence, the actual contribution of each Aurora kinase in obtaining the final cellular response has to be determined empirically for the different tumor types.

The clinical use of selective inhibitors of Aurora kinases is desirable as they might reduce adverse effects of the dual inhibitors, allowing the administration of higher doses and/or the prolongation of therapy. Recent phase I trials carried out with either MLN8237 or AZD1152 have shown tolerability and favorable pharmacokinetics in patients affected by advanced solid tumors, paving the way for future phase II experimentations (Boss et al. 2011, Cervantes et al. 2012, Dees et al. 2012, Schwartz et al. 2013). On the basis of results from a number of studies, both MLN8237 and AZD1152 deserve further investigation in in vivo systems to assess their therapeutic potential against solid tumors. However, on the basis of results from the present preclinical study, MLN8237 is as a more promising drug candidate for ATCs.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-14-0299.

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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