Cancer Science
The official journal of the Japanese Cancer Association

Evaluation of Aurora kinase inhibition as a new therapeutic strategy in anaplastic and poorly differentiated follicular thyroid cancer

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(Received September 1, 2010/Revised December 16, 2010/Accepted December 20, 2010/Accepted manuscript online January 8, 2011/Article first published online February 17, 2011)

Due to an unfavorable prognosis using the usual therapy, patients with anaplastic thyroid cancer (ATC) are in desperate need of new therapeutic strategies. The objective of this study was to evaluate the effects of MLN8054, an inhibitor of the Aurora serine/threonine kinases, on ATC cells *in vitro* and on ATC xenografts as a new therapeutic strategy for ATC. Three anaplastic (Hth74, C643, Kat4) and one follicular (FTC133) thyroid cancer cell lines were evaluated *in vitro* and Kat4 xenografts *in vivo*. The antiproliferative effect of MLN8054 (0.1–10 μM) on thyroid cancer cells was quantified by sulphorhodamine B-assay. The proapoptotic effect and the effects on the cell cycle were evaluated by flow cytometry after Annexin-V-FITC staining. Further Histone H3 phosphorylation was analysed. *In vivo*, antiproliferative and antiangiogenic effects were assessed by tumor volume and morphometric analysis following immunohistochemical staining (Ki-67, pHisH3, CD31). Treatment of the different TC cells with MLN8054 inhibited proliferation in a time- and dose-dependent manner, with IC_{50} values between 0.1 and 10 μM. Administration of MLN8054 resulted in an increase of apoptotic cells, decreased Histone H3 phosphorylation and induced cell cycle arrest. *In vivo*, treatment of ATC by MLN8054 resulted in an up to 86% reduced tumor volume and 89% reduced tumor vascularity. In conclusion, our data demonstrated that Aurora kinase inhibition is effective in reducing cell growth and inducing apoptosis of ATC *in vitro* and tumor growth and vascularity *in vivo*. Controlled clinical studies on MLN8054 or comparable compounds would be worthwhile to evaluate its potential therapeutic value for treatment of ATC. (*Cancer Sci* 2011; 102: 762–768)

Thyroid cancer (TC) is the most common endocrine malignancy representing an increasing incidence.[1] Most of the thyroid malignancies are well differentiated and treatment by surgery and radioiodine results in long-term survival. However, dedifferentiation of a small number of these tumors is thought to give rise to anaplastic thyroid carcinoma (ATC), resulting in a fulminant course and fatal outcome.[2] Therapy strategies published to date are literally ineffective, confirming a desperate need for innovative and effective treatment for ATC.[3] Genetic alterations and dysfunction of signaling pathways identified in ATC offer the possibility of targeted therapies with so-called ''small molecule inhibitors.''[4,5]

Highly aggressive tumors like ATC are characterized by extensive proliferation, multinuclearity, increased DNA content and high transcription activity. Therefore, the involvement of Aurora kinases (AK) in cancer development is of broad interest as well as the potential of anticancer treatment by specific kinase inhibitors.[6,7] So far, only rare data focusing on the role of AK in TC are available and the clinical impact remains somewhat confused.

Aurora kinases are serine/threonine kinases and up to now three members of this protein family are known: Aurora A, B and C. Aurora kinases A and B are key regulators of mitosis, chromosome segregation and cytokinesis and are associated with the ERK/MAPK pathway.[8,9] They were found to be overexpressed in various human malignancies, including thyroid cancer.[10–12] However, the distinct function of Aurora C, which is able to form a complex with Aurora-B, INCENP and survivin in mitotic cells remains unclear.[11]

Aurora kinases A, B, and C were documented to be expressed in normal thyrocytes and thyroid cancers cells at the mRNA and protein level. However, overexpression of Aurora A and B appeared to be restricted to cancer cells.[11] In a series of anaplastic thyroid tumor samples Aurora A and C were found to be overexpressed.[4] In contrast, according to a study on TC cell lines, Aurora B overexpression was associated with an undifferentiated thyroid cancer phenotype.[13] Therefore, the suggestion of Aurora B to be a biomarker for ATC and a possible therapeutic target for therapy has to be taken with caution. Inhibiting effects targeting Aurora kinases were demonstrated preclinically by *in vivo* siRNA experiments and by blocking Aurora kinase activity in ATC cell lines using VX-680 as an inhibitor.[13,14] However, clinical data are not yet available. Nevertheless, a panel of Aurora kinase inhibitors entered clinical Phase I and II studies, including various tumor entities demonstrating some encouraging results.[9] Currently, two trials are recruiting patients with 131I-refractory and medullary thyroid cancer for Aurora kinase inhibition. Among the currently evaluated AK inhibitors, MLN8054 is an orally active pyrimido-benzazepine that selectively inhibits Aurora A, but at higher concentrations was found also to inhibit Aurora B.[15,16] In this study, we therefore investigated the effects of MLN8054 treatment on thyroid cancer, particularly ATC, in a set of preclinical experiments. The data revealed, both *in vitro* and in a xenograft model, showed promising antitumor activity for MLN8054 in ATC.

Materials and Methods

Cell lines and culture conditions. Four TC cell lines, three anaplastic (Hth74,[17] C643,[17] Kat4[18]) and one follicular FTC133[19] were used. Cells were maintained in full growth medium (FGM) as described previously.[20] During experiments, FGM was changed to serum-starved conditions (2% FCS = DMEM-h21/Ham's F12 1:1 [v/v] supplemented with 2% FCS). Cell viability was assessed by trypan blue exclusion.

Drugs. MLN8054 (4-[(9-chloro-7-[2,6-difluorophenyl]-5H-pyrimido[5,4-d][2]benzazepin-2-yl)amino]-benzoic acid) was

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doi: 10.1111/j.1349-7006.2011.01853.x

kindly provided by Millennium Pharmaceuticals (Cambridge, MA, USA). For *in vitro* experiments, MLN8054 stock solutions (10 mM) were prepared in dimethylsulfoxide (DMSO). For *in vivo* experiments, MLN8054 was dissolved in 20% hydroxypropyl-β-cyclodextrin and 3.5% sodium bicarbonate (50:50).

Western blot analysis. Cells maintained in FGM and incubated with and without nocodazole (200 ng/mL) for 16 h were used for western blot analysis of Aurora A, B and C expression. For studying the *in vitro* effects of Aurora kinase inhibition, nocodazole-arrested cells (200 ng/mL, 16 h) were treated with increasing concentrations of MLN8054 (1, 10, 100 μM) for 2 h. Total cell lysates were prepared using RIPA (Santa Cruz, Heidelberg, Germany) or RIPA containing Phosphatase-Inhibitor-Cocktail (Calbiochem, San Diego, CA, USA) for phosphoproteins. Protein content was determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA) and western blot analysis was performed (for details see Supporting Information).

RNA preparation and RT-PCR for Aurora A, B and C. RNA was extracted from cells grown in FGM, treated with DNaseI and reverse transcribed into cDNA (Superscript III Reverse Transcriptase; Invitrogen, Grand Island, NY, USA). Semiquantitative RT-PCR was performed using ReadyMix Taq PCR Reaction Mix (Sigma, St Louis, MO, USA) and specific primers for Aurora A, B and C and β-microglobulin as described elsewhere.[11]

Cell proliferation assay. Antiproliferative activity of MLN8054 was evaluated using the SRB (sulphorhodamine B)-assay.[21] Briefly, cells were plated at a density of 1×10^4 cells into triplicate wells of 96-well plates. After 24 h the FGM was changed to 2% FCS and cells were treated with increasing concentrations of MLN8054 (0.01–10 μM) for up to 144 h with the medium changed after 72 h. Cells treated or untreated with DMSO served as controls. After 24, 72 and 144 h the cell density was determined by SRB-assay (OD 490 nm) and IC_{50} values were calculated. Experiments were repeated twice.

Cell cycle analysis. For cell cycle analysis the cells were plated at a density of 1×10^6 in six-well plates. After 24 h the FGM was changed to 2% FCS and MLN8054 was added to final concentrations of 1 and 10 μM for 24 and 48 h. Cells were stained with propidium iodide (PI) (50 μg/mL PI, 200 μg/mL RNase) for 15 min, analysed by flow cytometry (FACS) (BD LSRII cytometer, BD Franklin Lakes, NJ, USA) and the percentages of cells in G1, S and G2/M phase were determined using ModFit (Verity Software House, Topsham, ME, USA). Untreated and DMSO-treated cells were used as controls. Experiments were repeated twice.

Apoptosis analysis. Apoptosis was analysed by FACS (Annexin V-FITC) and measurement of caspase-3 activity. For FACS analysis the cells were plated into six-well plates, switched to 2% FCS after 24 h and incubated with MLN8054 (0.01–10 μM) for 24 and 48 h. The cells were then stained for Annexin V according to the manufacturer's instructions (Annexin V-FITC Apoptosis Detection kit [Alexis-Biochemicals, San Diego, CA, USA]). Briefly, cells were washed with binding buffer, stained with AnnexinV-FITC (1:40) for 10 min at room temperature, washed again with binding buffer, stained with PI (20 μg/mL) and analysed by cytometer. For analysis of caspase-3 activity the cells were plated in triplicate wells of 96-well plates (1×10^4 cells/well), switched to 2% FCS after 24 h and incubated with MLN8084 (0.01–10 μM, 24 h). Caspase-3 activity was assessed using a luminescence based caspase assay (Caspase GloTM 3/7 Assay, GloMax Multimode Reader [Promega, Mannheim, Germany]). Experiments were repeated twice.

***In vivo* animal experiment.** Six-week-old athymic nude mice (nu/nu) were purchased (Harlan Winkelman, Borchen, Germany) and allowed to adapt to the laboratory environment for 1 week. Experiments were performed as previously described.[22] Briefly, 3×10^6 cells (Kat4, anaplastic)/animal were immobilized in Matrigel (300 μg/mL) (BD Biosciences, San Jose, CA, USA) and xenotransplanted. After tumors reached 5 mm in diameter the mice were randomized into groups of 10 mice. MLN8054 (10 mg/kg, 5 days/week) was administered orally. Control groups were treated with vehicle or left untreated. Tumor volume (calculated as described elsewhere[23]), animal weight and side-effects were monitored weekly. After 4 weeks the mice were killed and tumor tissue was removed and processed for immunhistochemistry. All procedures were monitored and approved by the local ethics committee and federal authorities and conducted in accordance with the guidelines for the welfare of animals in experimental neoplasia.

Immunhistochemistry. Paraffin sections of 3 μm were used for immunhistochemical analysis. Staining procedures and antibodies are described in detail in the Supporting Information.

Morphometrical analysis was done using the Leica imaging system QWin (Leica, Wetzlar, Germany). The Ki-67 staining index was determined by evaluating the number of positive-stained cells per high-power field (×40) in 10 random areas within the maximal tumor cell load. Tumor angiogenesis was quantified following CD31 staining by assessment of the vascular surface density (VSD, 1 per millimeter) as described earlier.[22,24] Cleaved caspase-3 and phospho HistonH3 (pHisH3) staining was quantified by assessment of positive-stained areas in 10 random areas (×40) within the maximal tumor cell load.

Results

Expression of Aurora A, B and C. Semiquantitative RT-PCR analysis revealed the expression of all three members of the Aurora kinase family in the TC lines under investigation (Fig. 1A). In contrast, at protein level Aurora A was detectable only in FTC133 (follicular) and Kat4 (anaplastic) cells. Expression was found to be lower than in HeLa cells, which were used as a positive control. As in Hela cells nocodazole treatment

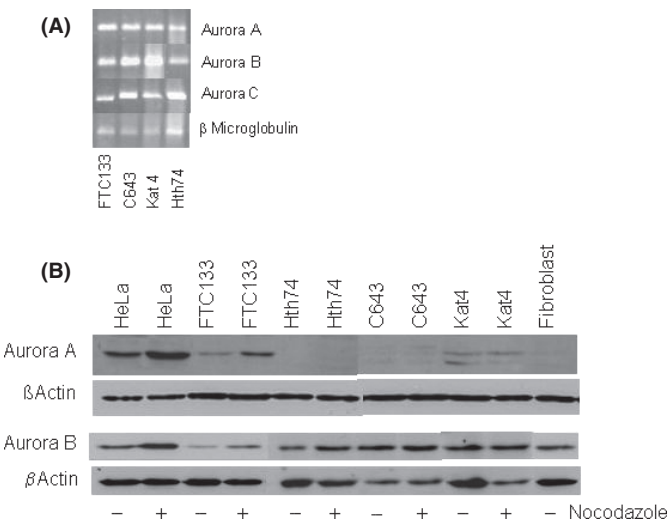

Fig. 1. Analysis of Aurora kinase expression in different thyroid cancer (TC) cell lines (FTC133 [follicular], Kat4, Hth74 and C643 anaplastic) on the mRNA level (A) and protein level (B). (A) Expression of Aurora A, B and C mRNA demonstrated by RT-PCR. β-Microglobulin as a loading control. (B) Western blot analysis of different TC cell lines incubated with and without nocodazole (200 ng/mL) for 16 h compared with HeLa and fibroblast cells (Aurora A, skin fibroblast CCD-1064Sk; Aurora B, human embryo fibroblast NIH3T3). 10% SDS-PAGE. Protein load: 70 μg Aurora A; 50 μg Aurora B. Membranes were probed for Aurora A (ARK1 [N-20]) and Aurora B (AIM-1). β-Actin was the loading control.

resulted in elevated protein expression. Aurora B expression on the other hand was confirmed by western blot analysis for all cell lines. The expression level was found to be somewhat lower than in HeLa cells and was affected by nocodazole treatment as well (Fig. 1B).

MLN8054 inhibits proliferation of cultured TC cells. To evaluate the effect of MLN8054 on proliferating TC cells proliferation assays were carried out using the SRB assay for assessment of cell density. From these experiments a time and dose-dependent inhibition of cell proliferation was shown (Fig. 2). Antiproliferative effects were most pronounced in FTC133 and Hth74 cells. Here, cell numbers were reduced by 82% and 80%, respectively (10 μM MLN8054, 144 h), compared with 67% and 34% in Kat4 and C643 cells. IC_{50} values at 144 h were calculated as 0.081 ± 0.007 μM (FTC133), 0.098 ± 0.011 μM (Hth74), 0.82 ± 0.31 μM (Kat4) and >10 μM in C643 cells.

MLN8054 treatment results in inhibition of Aurora kinase activity. To evaluate the effect of MLN8054 on the activity of Aurora kinases, nocodazole-arrested cells were incubated with increasing concentrations of MLN8054 and analysed by western blotting using an antibody specific for Aurora A, B and C, phosphorylated at Thr288, Thr232 or Thr198, respectively. Basal (FGM) phospho-Aurora B was displayed in Kat4 and FTC133 cells, whereas in C643 and Hth74 cells it was hardly detectable. In all cell lines Aurora B activity was found to be enhanced distinctly by nocodazole treatment and was inhibited in a dose-dependent manner by MLN8054 (Fig. 3A) without affecting total Aurora B (an example is given in Fig. 3B).

Phospho-Aurora A in contrast was hardly detectable in any cell line, even in the nocodazole-treated cells. Here, extremely weak signals were found for FTC133, C643 and Hth74 cells, which disappeared under MLN8054 treatment (Fig 3A, arrows). But nevertheless, when compared with HeLa cells, Aurora kinase A activity seems to be negligible in the TC cells investigated. In addition, Aurora C kinase, phosphorylated on Thr198, was found in all cell lines and was inhibited by MLN8054 in a dose-dependent manner. The inhibiting effects on Aurora B and C were most pronounced in Hth74 cells, whereas FTC133 cells displayed were less sensitive (Fig. 3A).

MLN8054 treatment results in G2/M accumulation. To evaluate the effect of MLN8054 on the cell cycle, DNA profiles were created by FACS and cells in G1, S and G2/M phase were calculated in percentages. In all cell lines, treatment with 1 and 10 μM MLN8054 for 24 and 48 h induced a dose-dependent accumulation of cells in G2/M relative to the control cells (Fig. 4, Table 1). The G2/M arrest induced was characterized by accumulation of up to 50% cells in the G2/M phase after treatment with 10 μM MLN8054 for 24 h, compared with values <10% in the control cells. A further increase in G2/M accumulation was observed after 48 h. This effect was most pronounced in FTC133 (≈66%) and Kat4 (≈61%) cells (Table 1).

MLN8054 induced apoptosis in TC cell lines. The effect of MLN8054 on tumor cell apoptosis was first examined by Annexin V-FITC staining and FACS. Following MLN8054 treatment (10 μM MLN8054, 24 h) the number of apoptotic cells was increased up to 5% compared with values <1% in control samples (2% FCS) Additionally, the number of dead cells increased with time and concentration, implying a cytotoxic effect at higher concentrations.

Induction of apoptosis was further investigated by determination of caspase-3 activity, which was distinct in Kat4 cells

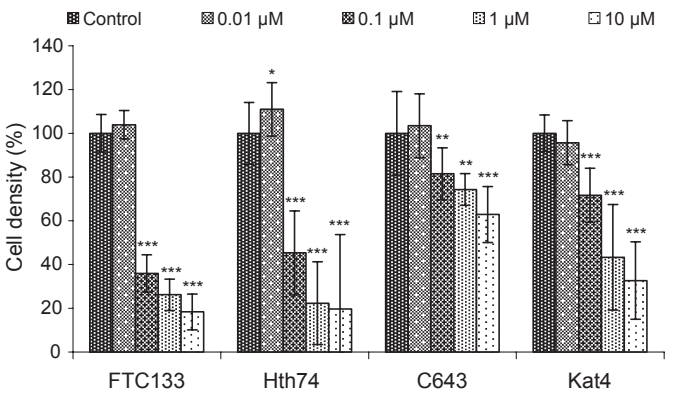

Fig. 2. Dose response curves for growth of thyroid cancer (TC) cell lines (FTC133 follicular, Hth74, C643 and Kat.4.1 anaplastic) incubated with increasing concentrations of MLN8054 (0.1–10 μM) in serum-starved medium for 144 h. Data reported represent the mean ± SD of three independent experiments with triplicates of each. Control: DMSO-treated cells.*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ as revealed by the Wilcoxon rank-sum test.

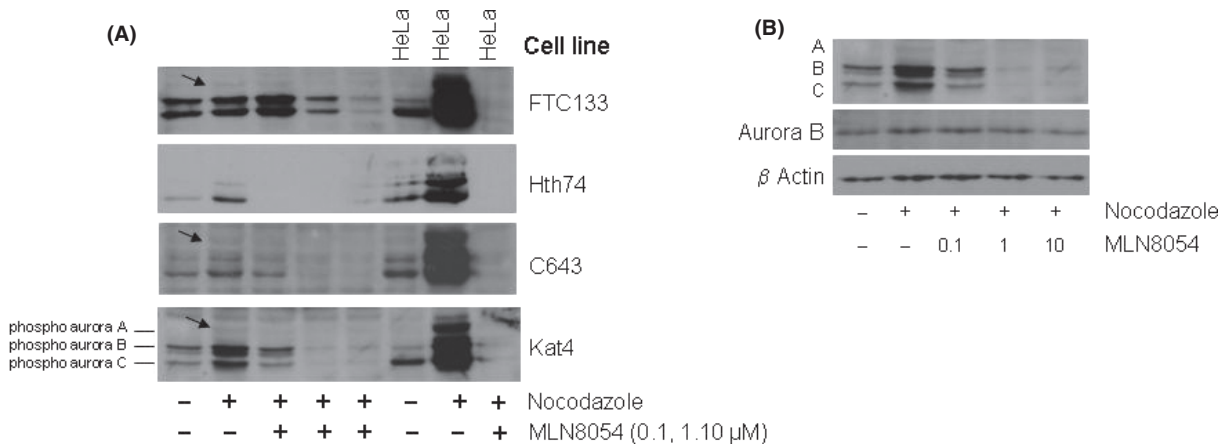

Fig. 3. Effects of MLN8054 on Aurora kinase activity (A) and total Aurora kinase B (B) as revealed by western blotting. (A) Western blot analysis (10% SDS-Page, 70 μg protein/lane) of thyroid cancer (TC) cells incubated with and without nocodazole and treated with increasing concentrations of MLN8054 as indicated and probed with an anti-phospho Aurora A, B and C antibody. For comparison, the results revealed for HeLa cells (positive control) are shown. Equal loading was confirmed by β-actin staining (data not shown). Expression of Aurora A is marked by arrows. (B) Western blot (10% SDS-Page, 50 μg protein/lane) of Kat4 cells treated as indicated under (A) and probed with an anti-Aurora B antibody. Corresponding β-actin staining.

doi: 10.1111/j.1349-7006.2011.01853.x

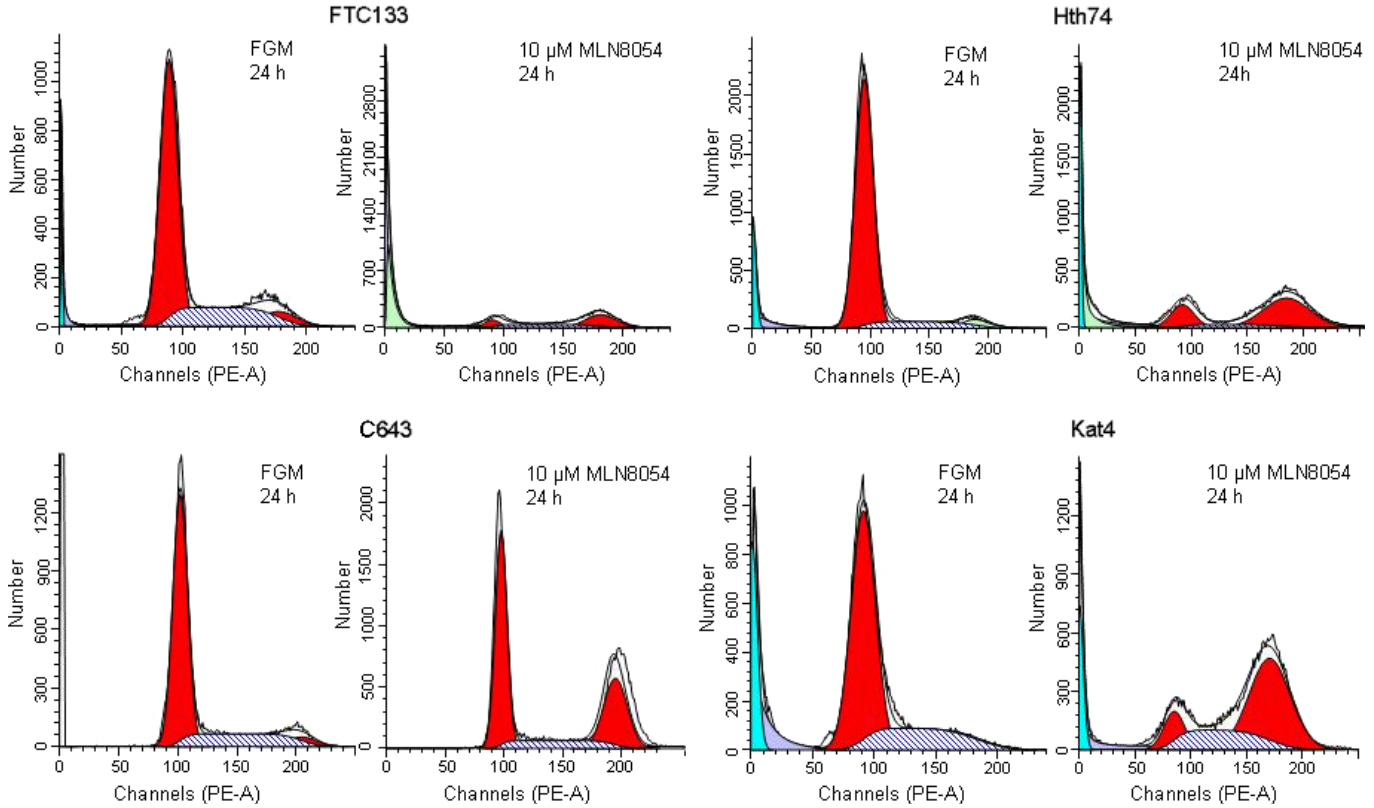

Fig. 4. G2/M arrest induced by MLN8054 treatment. DNA profile of untreated and MLN8054-treated (10 μM, 24 h) thyroid cancer (TC) cells (follicular and anaplastic) as evaluated by FACS.

Table 1. Induction of G2/M-arrest in different thyroid cancer cell lines by MLN8054

Cell line	FTC133		HTh74		C643		Kat 4	
Phase of cell cycle	G1	G2	G1	G2	G1	G2	G1	G2
FGM, 24 h	79.3 ± 0.2	0.0 ± 0	82.8 ± 8.4	2.8 ± 1.8	69.3 ± 4.4	7.9 ± 5.3	68.6 ± 0	8.3 ± 0.9
1 μM, 24 h	46.8 ± 37.9	22.3 ± 27.9	47.8 ± 33.3	43.2 ± 41.7	28.6 ± 14.4	51.6 ± 1.7	20.9 ± 8.2	49.5 ± 14.7
10 μM, 24 h	22.0 ± 9.4	46.2 ± 8.8	25.0 ± 0.7	49.1 ± 20.9	48.6 ± 4.8	38.5 ± 6.6	19.6 ± 9.2	51.7 ± 14.8
FGM, 48 h	54.1 ± 32.4	0.0 ± 0	88.4 ± 16	1.5 ± 2.1	75.0 ± 3.3	8.4 ± 4.3	79.5 ± 1	2.7 ± 3.8
1 μM, 48 h	40.6 ± 41.4	27.9 ± 39.5	42.7 ± 41.6	28.9 ± 23	24.2 ± 5.2	66.0 ± 10.7	33.0 ± 7.3	57.5 ± 5.5
10 μM, 48 h	21.2 ± 10.2	66.4 ± 5.1	29.9 ± 0.2	48.1 ± 3.8	35.2 ± 18.3	53.0 ± 26.4	26.9 ± 12.2	60.7 ± 12.8

Numbers represent the means ± SD of two experiments. FGM, full growth medium.

followed by FTC133 cells and was comparatively low in Hth74 and C643 cells. By MLN8054 treatment, caspase-3 activity was increased dose-dependently in FTC133, Hth74 and C643 cells (Fig 5A).

MLN8054 treatment reduced phosphorylation of Histone H3. Due to the induction of a mitotcic arrest, an increase of pHisH3 was demonstrated for ATC cells by western blotting. The phosphorylation of HisH3 was downregulated by MLN8054 at concentrations as low as 0.1 μM (Hth74) and 1 μM (C643, Kat4, FTC133). The follicular FTC133 cells proved to be the least sensitive (Fig. 5B). Considering dephosphorylation of pHisH3 as an Aurora B kinase effect, MLN8054 seems to inhibit Aurora B activity in TC.

***In vivo* evaluation of MLN8054 effects.** *MLN8054 treatment results in growth inhibition of TC cell xenografts.* To evaluate the potential of MLN8054 in suppressing tumor growth *in vivo*, the effect of MLN8054 on xenotransplanted ATC cells was explored. By application of MLN8054 (10 mg/kg, 5 days/week) tumor growth was inhibited significantly (Fig. 6A; $P = 0.0087$). Tumor volumes were reduced by 85% compared with the vehicle-treated control group. Animal weight remained almost constant and no side-effects were observed.

MLN8054 treatment results in decreased cell proliferation, angiogenesis and Histone H3-phosphorylation and increased Caspase-3 activity in vivo. As revealed by Ki-67 immunhistochemistry, MLN8054-dependent tumor growth inhibition *in vivo* was accompanied by a significantly reduced number of proliferating cells (Fig. 6B). In the treatment group, 8.2 ± 3.6% cells were stained positively compared with 34.5 ± 2.2% in the vehicle-treated group. Based on this data, MLN8054 was calculated to reduce proliferative activity approximately 72% in experimental ATC tumors.

MLN8054 also exhibited potent antiangiogenic activity *in vivo*. Vascular surface density (VSD), as revealed by CD31

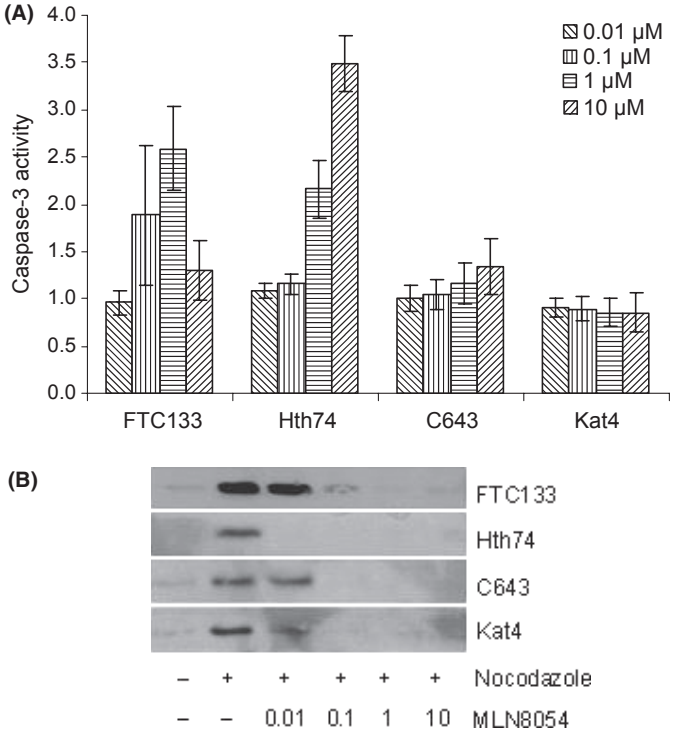

Fig. 5. Effects of MLN8054 on caspase-3 activity (A) and Histone H3-phosphorylation (B) *in vitro*. (A) Caspase-3 activity of thyroid cancer (TC) cells incubated with increasing concentrations of MLN8054 (0.01–10 μM) for 24 h. Depicted x-fold increase compared with the control (DMSO). Mean ± SD of two experiments with triplicates of each. (B) Western blots (10% SDS-Page, 30 μg protein/lane) of lysate of nocadazole-arrested and MLN8054-treated TC cells.

staining and morphometrical analysis, was significantly reduced in the treatment group compared with the vehicle-treated group (Fig. 6C). Expressed as percentage, VSD was reduced at approximately ≈90%.

To evaluate the effect of Aurora kinase inhibition on apoptosis, tumor samples were stained for cleaved caspase-3. Assessment of positively stained areas displayed a twofold increase in the MLN8054 group compared with the vehicle-treated group (Fig. 6D). Therefore, induction of caspase-dependent apoptosis can be assumed.

Tumor growth inhibition induced by MLN8054 was further paralleled by reduction of pHisH3 staining of approximately 94% compared with the vehicle-treated group (Fig. 6E). Because a decrease in pHisH3 is associated with the phenotype of Aurora B inhibition, this indicates an Aurora B inhibiting effect for MLN8054.

Discussion

Failure in mitosis leading to genomic instability is known to be involved in tumor development. Together with an increased understanding of the mitotic process, this contributed to the development of new classes of compounds apart from the classic antimitotics like taxanes or vinca alkaloids over last 10 years. Among these, the Aurora kinase inhibitors raised the possibility to directly influence different steps in the mitotic process. Facing the fact that anaplastic thyroid cancer is characterized by excessive mitotic activity and cellular proliferation, this class of drugs raises new hope concerning the treatment of this fatal disease. Based on results published by Wiseman *et al.*,[4] who analyzed a clinical series of ATC and demonstrated Aurora A to be expressed by 83%, Aurora C by 90%, but Aurora B only by 13%, we focused this investigation on Aurora A inhibition choosing MLN8054 as a so-called Aurora A selective inhibitor. Consistent with the findings on colorectal and prostate cancer,[16] we could demonstrate antiproliferative *in vitro* and *in vivo* effects of MLN88054 on thyroid cancer cells. However, Aurora B and C seem to be predominant in the cell lines used in this investigation and MLN8054 is proven to display inhibiting activity to them.

The expression of Aurora A, B and C mRNA, documented for the TC cell lines used, is consistent with a study of Ulisse *et al.*[11] However, at protein level expression of Aurora A could be confirmed only for FTC133 and Kat4 cells. Compared with HeLa cells, Aurora A expression was distinctly less. Therefore, it can be stated that Aurora A is not generally overexpressed in TC, especially in ATC cells. Aurora kinase B on the other hand was found to be expressed strongly in all cell lines, a fact that has been demonstrated for ATC cell lines previously.[13] Overexpression of Aurora B has been described to be associated with highly aggressive, dedifferentiated human tumors.[12,25–29] In addition, it correlates with increased Ki-67 expression,[26] expression of survivin and Cyclin A,[30] as well as distinct histological features like poor differentiation and multinuclearity. Although the detailed effect of Aurora C on tumor proliferation is not clear, it is known that aurora kinase C can reconstitute Aurora kinase B function in Aurora kinase B-depleted cells, indicating that Aurora kinase C can carry out mitotic functions.[11]

By evaluating the *in vitro* effect of MLN8054 on our TC cell lines, we could demonstrate a time and dose-dependent inhibition of tumor cell number with IC_{50} between 0.1–1 μM and >10 μM for the anaplastic cell line C643. Except for the latter, these values are in line with results reported for various other solid tumor cell lines.[16]

When the effect of MLN8054 (0.01, 1 and 10 μM) on Aurora kinase phosphorylation was revealed by western blot analysis, complete suppression of activity could be demonstrated for Aurora B and C; Aurora A activity, on the contrary, was hardly detectable with western blotting, even following nocodazole treatment, although total Aurora A was demonstrated in FTC133 and Kat4 cells. MLN8054, originally described to selectively inhibit Aurora A, was also documented to inhibit Aurora B (IC_{50} Aurora A 0.004, Aurora B 0.172 μM) by Manfredi *et al.*[16] Therefore, inhibition of Aurora B activity, indicated by decreased phosphorylation on Thr 232, seems to be the main reason for the inhibiting effect of MLN8054 on the TC cells used in our investigations. This might be underlined by the fact that Aurora B was found to be predominant over Aurora A in the TC cells used. Regarding Aurora kinase C, this is, to the best of our knowledge, the first time that an Aurora C kinase inhibiting effect was demonstrated for MLN8054. Aurora kinase C was believed to be restricted primarily to the testis, but recently it has been shown to be a chromosomal passenger protein and to regulate mitotic chromosome dynamics in accordance with Aurora B.[31,32] Nevertheless, the distinct function of Aurora C in thyroid cancer remains to be elucidated. In conclusion, the TC cells used in this investigation seem to be affected by MLN8054 mainly through Aurora B and possibly also through Aurora C inhibition, whereas inhibition of Aurora A seems to play a minor role.

MLN8054 treatment resulted in accumulation of TC cells in the G2/M phase of the cell cycle, which was also shown in colon and prostate cancer cells and was described together with increased aneuploidy to be characteristic for Aurora kinase inhibition by MLN8054.[16] The induction of apoptosis was assessed by two different methods; here the effect of MLN8054 was found negligible (Annexin V-FITC staining) or moderate (caspase-3 induction). Depending on the cell line, caspase-3 activity

doi: 10.1111/j.1349-7006.2011.01853.x

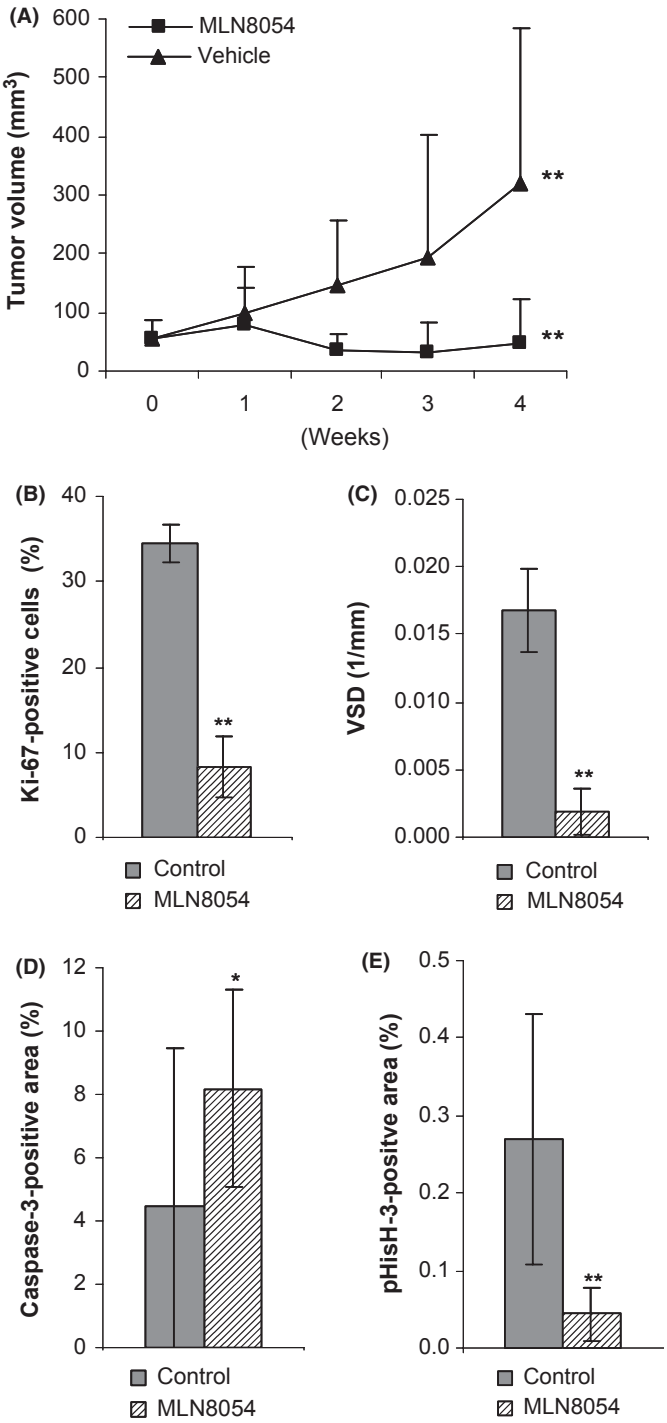

Fig. 6. Effects of MLN8054 on *in vivo* growth of thyroid cancer (TC) cells (A) and immunhistochemical analysis of tumor samples concerning proliferation (B), angiogenesis (C), apoptosis (D) and pHisH3 (E). (A) Time course of tumor development of xenotransplanted Kat4 cells (anaplastic) treated with MLN8054 (10 mg/kg, oral application, 5 days per week) over 4 weeks. Tumor volumes (TV) calculated as TV = (l × b2) × 0.5. Means ± SD of tumor volume for each group (n = 10) are depicted. (B) Rate of proliferation as revealed by Ki-67 immunostaining and counting Ki-67 positive cells in 10 fields (×40) per slide. Means ± SD of Ki-67 positive cells depicted for treated and control groups (n = 10 each). (C) *In vivo* effects of MNL8054 on angiogenesis as revealed by measurement of vascular surface density (VSD) following CD31 immunostaining. (D) Means ± SD of caspase-3 positivity for the treated and control groups (n = 10). Ten fields (×40) per slide analysed. (E) Means ± SD of the pHisH3-positive area are depicted for the treated and control groups (n = 10). Ten fields (×40) per slide were analysed. *P < 0.05. **P < 0.001. An extension of the nonparametric Kruskal–Wallis test was used to calculate the P-value for differences in the tumor volume growth pattern between the MLN8054 and vehicle groups. The Wilcoxon rank-sum test was used to compare the percentage of Ki-67 positive cells, VSD and caspase-3-positive and pHisH3-positive areas in percentage, respectively, between the MLN8054 and control groups.

was increased up to 3.5-fold. Similar results were obtained using VX680, a pan-Aurora kinase inhibitor, in ATC.[14]

Application of Aurora kinase inhibitors typically results in decreased phosphorylation of Histone H3. According to Harrington *et al.*[33] this is consistent with Aurora B inhibition and it was documented for all our TC cells exposed to MLN8084. Facing the fact that DNA-associated Histone H3 is a target of Aurora B and overexpression of Aurora B is accompanied by a dramatic increase of pHisH3, it can be assumed that MLN8054, considered to be an Aurora A inhibitor, also inhibits Aurora B. Decreased pHisH3, as documented in the present study, further supports the statement given concerning the inhibiting effect of MLN5084 on Aurora B in thyroid cancer cells.

When MLN8054 was administered to nude mice bearing ATC-xenografts, a strong inhibition of tumor growth could be demonstrated. Moreover, in some cases a complete tumor regress was seen. By analyzing the effects of Aurora kinase inhibition in the tumor samples, the results of the *in vitro* experiments could be widely confirmed. A dramatic inhibition of tumor cell proliferation, indicated by decreased Ki-67-positive cells, was observed. This was paralleled by a decrease of pHisH3-positive tumor areas, suggesting Aurora B inhibition to be a major effect in this context. In addition, a moderate induction of apoptosis occurred, documented by increased cleaved caspase-3. For MLN8054, this has been demonstrated for human colon cancer xenografts elsewhere.[16] Because MLN8054 was proven to induce p73-mediated apoptosis in p53-deficient cancer cells, a fact known to be frequent in ATC, this also could be a possible explanation in this context.[34] Moreover, a strong decrease in tumor vascularity was seen, implicating an antiangiogenic effect of MLN8054, as it has been suggested for other Aurora kinase inhibitors like CYC116 or ENMD-2076. They were shown to not only inhibit Aurora A selectively over Aurora B, but also to inhibit a number of kinases important for tumor growth and, in particular, growth factor receptors critical to angiogenesis.[35] The effects of Aurora kinase targeted therapy on ATC *in vivo*, presented in the current study, are promising and stand in line with the encouraging results found in other solid human tumor xenograft models like colon, lung, ovarian, hepatocellular and hematological tumors.[36–38] Results of clinical phase 1 trials using Aurora kinase inhibitors published to date demonstrate that the dose-limiting toxicity is neutropenia and only limited nonhematological toxicity occurred.[39,40] Results on response rates to Aurora kinase inhibition can be expected by ongoing phase 2 trials (http://clinicaltrials.gov).

Hence, our preclinical data suggests that Aurora kinase inhibition seems to be a promising new therapeutic option for patients suffering from ATC. By demonstrating inhibiting effects of MLN8054 apart from Aurora A on Aurora kinases B and C in TC, MLN8054 might be considered as an Aurora A kinase inhibitor with relevant activity on Aurora B and C. Clinical investigations, using MLN8054 or second generation compounds of the same class alone or in combination with inhibitors targeting different pathways associated with ATC, may be a promising field of research to find therapeutic armamentarium against the, today still dismal, ATC.

Acknowledgment

This work was supported by a grant from the Wilhelm Sander Foundation (grant number 2008.088.1).

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Appendix to the methods of western blotting.

Data S2. Appendix to the methods of immunohistochemistry.

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doi: 10.1111/j.1349-7006.2011.01853.x