

The aurora kinase inhibitor VX-680 shows anti-cancer effects in primary metastatic cells and the SW13 cell line

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Abstract New therapeutic targets are needed to fight cancer. Aurora kinases (AK) were recently identified as vital key regulators of cell mitosis and have consequently been investigated as therapeutic targets in preclinical and clinical studies. Aurora kinase inhibitors (AKI) have been studied in many cancer types, but their potential capacity to limit or delay metastases has rarely been considered, and never in adrenal tissue. Given the lack of an effective pharmacological therapy for adrenal metastasis and adrenocortical carcinoma, we assessed AKI (VX-680, SNS314, ZM447439) in 2 cell lines (H295R and SW13 cells), 3 cell cultures of primary adrenocortical metastases (from lung cancer), and 4 primary adrenocortical tumor cell cultures. We also tested reversan, which is a P-gp inhibitor (a fundamental efflux pump that can extrude drugs), and we measured AK expression levels in 66 adrenocortical tumor tissue samples. Biomolecular and cellular tests were performed (such as MTT, thymidine assay, Wright's staining, cell cycle and apoptosis analysis, Western blot,

qRT-PCR, and mutation analysis). Our results are the first to document AK overexpression in adrenocortical carcinoma as well as in H295R and SW13 cell lines, thus proving the efficacy of AKI against adrenal metastases and in the SW13 cancer cell model. We also demonstrated that reversan and AKI VX-680 are useless in the H295R cell model, and therefore should not be considered as potential treatments for ACC. Serine/threonine AK inhibition, essentially with VX-680, could be a promising, specific therapeutic tool for eradicating metastases in adrenocortical tissue.

Keywords VX-680 · Aurora kinases · Adrenal gland · Metastasis · Reversan · SW13 cells · Primary cell cultures · Lung carcinoma

Abbreviations

AK	Aurora kinase
AKI	Aurora kinase inhibitors
ACT	Adrenocortical tumors
ACC	adrenocortical carcinomas
NACA	non-aldosterone-secreting cortical adenomas
APA	aldosterone-producing adenomas
NSCLC	non-small-cell lung cancer
<i>AURK</i>	AK gene

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Introduction

Aurora kinases (AK) are serine/threonine kinases fundamental to mitosis and cell division. The Aurora kinase family comprises 3 members, Aurora A, B and C, which have 67–76 % of their amino acid sequence in the catalytic domain in common [1]. AK are frequently found overexpressed in a variety of human tumors. In particular, Aurora kinase A is aberrantly

expressed in renal, ovarian, and hepatic carcinoma, and many others. As Aurora family members have been shown to interact with crucial tumor suppressor proteins and oncogenic proteins, they are also considered an important target for antitumor therapy [2]. Their importance in cancer has led to many compounds being designed to inhibit AK. Among them, VX-680 (also called MK-0457) has been shown to block cell cycle progression and induce apoptosis in many tumors, fostering growth inhibition in *in vivo* xenograft models [3]. SNS314, another AK inhibitor (AKI), has also exhibited a promising reduction in cell growth and tumorigenicity in *in vitro* experiments. ZM447439 is a selective, ATP-competitive inhibitor of Aurora kinases A and B [3] that was recently assessed in a primary cell culture obtained from a pediatric adrenocortical carcinoma, producing interesting results [4]. Reversan is a small molecule that selectively and non-toxicly targets P-glycoprotein (P-gp), a member of the superfamily of human ATP-binding cassette (ABC) proteins, which are fundamental efflux pumps in many tissues and have a protective role against xenobiotics [5]. P-gp is also implicated in multidrug resistance (MDR), a phenomenon that confers tumor cell resistance against a wide variety of anti-cancer drugs (such as vinca alkaloids, doxorubicin, etoposide) [6]. H295R and SW13 cells, and the majority of adrenocortical carcinomas (ACC) were recently found to express P-gp [7].

Virtually all types of tumor can develop metastases, which may spread to different tissues and organs via organ-specific colonization processes [8]. Metastases to the adrenal glands are rare, but may derive from various primary cancers, non-small-cell lung cancer (NSCLC) being considered the most likely [9]. The American Association of Endocrine Surgeons strongly recommends treating metastases as soon as possible with chemotherapy or surgical resection (adrenalectomy) to avoid exacerbating the patient's condition and symptoms [10].

Primary adrenocortical tumors (ACT), on the other hand, can be roughly divided into aldosterone-producing adenomas (APA), non-aldosterone-secreting cortical adenomas (NACA), and adrenocortical carcinomas (ACC). ACC are rare malignancies with an estimated incidence of 0.7–2.0 cases per million population a year [11]. They carry a poor prognosis, with a 5-year survival rate of less than 35 %, and metastases are common [12]. Surgery remains the mainstay of treatment, while pharmacological therapy is mainly based on the adrenotoxic drug, mitotane (o,p'-DDD), despite its low response rate and significant toxicity [13] – hence the need to develop new treatment options.

This study analyzed the expression of Aurora kinases A, B and C in 70 adrenal samples, 2 cell lines (SW13 and H295R, the 2 most frequent cancer cell models considered in the setting of adrenocortical diseases), 3 cell cultures from primary adrenocortical metastases (from NSCLC), and 4 primary adrenocortical tumor cell cultures. Three different AKI were

assessed and the origins of their cytostatic and cytotoxic effects on the cell models were investigated.

Materials and methods

Materials

VX-680, SNS314 mesylate, ZM447439, and mitotane were purchased from Selleckchem Europe. Fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), trichloroacetic acid (TCA), reversan and propidium iodide were purchased from Sigma Aldrich, Italy. DMEM-F12, 0.05 % trypsin-EDTA, insulin, transferrin, selenium and antibiotics were obtained from Life Technologies, Italy. The primary antibodies were: Akt (code 9272), phospho-Akt (Ser473) (code 9271), Erk1/2 (code 4695), phospho-Erk1/2 (Thr202/Tyr204) (code 4370), phospho-Aurora A (Thr288) (code 3079), phospho-Aurora A (Thr288) / phospho-Aurora B (Thr232) / phospho-Aurora C (Thr198) (code 2914), Aurora A (code 4718), Aurora B (code 3094), all from Cell Signaling Technology; caspase 3 (code 110,543) and 9 (code 112,888) from GeneTex; and anti- β -actin antibody (code A5441) from Sigma-Aldrich. The secondary antibodies were: horseradish peroxidase-labeled goat anti-mouse or anti-rabbit (Jackson ImmunoResearch).

Patients

Adrenocortical tissues were obtained from 23 patients with ACC, 26 with NACA, 17 with APA, and 9 individuals with normal adrenals (NA). The tissues were used for mRNA (RT-PCR) and DNA (PCR and sequencing) extraction. The etiology, clinical diagnosis and staging of each adrenocortical mass were established as explained elsewhere [14]. All clinical data are summarized in Table 1. All patients gave their written informed consent to the collection and use of their adrenal tissue for research purposes. The study was approved by the local ethics committee “Azienda Ospedaliera di Padova Prot. n.2989P/13”. It was conducted in accordance with the Declaration of Helsinki, and no children (under 18 years old) were involved in the study.

Cell cultures

The H295R and SW13 tumor cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The H295R cells came from a female patient diagnosed with an adrenocortical carcinoma. This strain secretes mineralocorticoids, glucocorticoids and adrenal androgens. The SW13 cells came from a metastatic deposit in the adrenal cortex derived from a lung carcinoma in a 55-year-old female [15]. Seven primary cell cultures were also studied: 3 cell

Table 1 Adrenocortical tumor patient clinical data ($N = 66$)

Parameter	Value
<i>Adrenocortical carcinomas (ACC)</i>	23
Female/male	14/9
Median age (range)	52 yr. (1–68 yr)
Function	
Cortisol producing	5
Cortisol and androgen producing	5
Aldosterone-producing	0
Androgen producing	0
Non-functioning	13
Median tumor size (range)	80 ± 17 mm
Tumor stage (ENS@T stage)	
I	0
II	2
III	1
IV	20
<i>Aldosterone producing adenoma (APA)</i>	17
Female/male	9/8
Median age (range)	53 yr. (22–77 yr)
Function:	
aldosterone-producing	17
<i>Non-aldosterone secreting cortical tumors (NACA)</i>	26
Female/male	18/8
Median age (range)	48 yr. (21–80 yr)
Function:	
Cortisol producing	17
Cortisol and androgen producing	3
Androgen producing	0
Non-functioning	6

cultures from primary adrenocortical metastases (derived from NSCLC) and 1 ACC, 1 APA, 2 NACA cell cultures. All experiments involving cell manipulation were conducted after incubating the cell lines with 0.1 % FBS as previously reported [16].

MTT assay and drug combination strategy

SW13 and H295R cells were plated in 96-well plates at a density of 5×10^3 cells/well in supplemented medium, with or without drugs. VX-680 was used at 1, 10, 50, 100, 200, 500 nM for 24 h, 48 h and 72 h (and also 120 h and 144 h for the H295R cells). SNS314 was used at 1, 10, 100 nM for 24 h and 72 h. ZM447439 was used at 0.1, 1, 10 μ M for 24 h and 72 h. Mitotane was used at 10 μ M for 24 h and 72 h. Methods and cell survival percentages were as previously reported [16]. Each analysis was run in quadruplicate and repeated 3 times. The half maximal inhibitory concentration (IC₅₀) was calculated in dose-response curves using CalcuSyn software

(Biosoft, Ferguson, MO). MTT assay was also performed on 4 primary adrenocortical tumor (1 ACC, 1 APA, 2 NACA) and 3 metastatic cell cultures. The concentrations of VX-680 were 10 and 200 nM for 24 h.

Interaction between VX-680 and mitotane was analyzed using the combination index (CI) values calculated with the CompuSyn 3.0.1 analysis software. CI values were generated over a range of fraction-affected levels from 5 %–95 % of growth inhibition, relying on the dose-response curves by MTT assay, as previously described [17]. Antagonism is defined as $CI > 1.15$, synergism as $CI < 0.85$ and additivity when the values lie between 0.85 and 1.15. Reversan was dissolved in DMSO (10 mg/ml) at 60 °C according to the manufacturer's instructions, and diluted to obtain a concentration of 1 mM.

[³H] thymidine assay

H295R and SW13 cells were seeded in 24-well plates and incubated with varying concentrations of VX-680 (10 and 200 nM) for 24 h and 72 h according to the method already described [18]. Experiments were performed in quadruplicate and repeated 3 times. [³H] thymidine assay was also performed on 2 primary adrenocortical tumor cell cultures (NACA-1 and APA). The concentrations of VX-680 used were 10 and 200 nM for 24 h; and the experiments were performed in triplicate.

Cell morphology assessment with Wright's staining

H295R and SW13 cells were cultured on coverslips for 48 h, incubated overnight in 0.1 % FBS, and then treated with VX-680 at 200 nM, SNS314 at 100 nM, ZM447439 at 10 μ M for 72 h and 144 h. Lower concentrations of the above-mentioned drugs were used too (data not shown). Then SW13 and H295R cells were treated as previously described [19]. Cell morphology was assessed by light microscopy at $\times 400$ magnification. The experiments were repeated twice.

Cell cycle distribution and apoptosis analysis

For the cell cycle analysis, the H295R cells were incubated for 24 h, 72 h and 144 h, and the SW13 cells for 24 h and 72 h. Briefly, H295R and SW13 cells were plated at a density of 1×10^6 cells/well and treated with VX-680 (10 and 200 nM) as previously described [17].

Apoptosis was assessed with the Annexin V-FITC kit (BD Biosciences, San Diego, CA, USA) as previously described [17]. Briefly, the H295R and SW13 cells were plated at a density of 1×10^6 cells/well for two days, then treated with VX-680 (10 and 200 nM) for 24 h and 72 h (and also 144 h for the H295R cells). The experiments were performed in

triplicate and data were assessed with the CXP analytical software.

Western blot analysis

The SW13 and H295R cells were treated with different concentrations of VX-680 (10 and 200 nM) for 72 h as previously described [20, 21]. Briefly, the proteins were extracted, loaded onto SDS/PAGE, electroblotted and incubated overnight with different primary antibodies. After adding secondary antibodies, immunoreactivity was detected with LiteAblot Extend Long-Lasting Chemiluminescent Substrate (EuroClone). Films were scanned and band intensity was quantified with ImageJ software 1.44p. The experiments were performed in triplicate.

Aurora kinases A, B and C mutation analysis and real-time PCR assay

AURKA and *AURKB* DNA were extracted from H295R and SW13 cells using genomic DNA isolation (QIAamp DNA micro Kit, Qiagen) according to the manufacturer's protocol. DNA was amplified using appropriate primers for *AURKA* [22] and *AURKB* [23], then assessed by direct sequencing (bidirectionally), as described elsewhere [24]. No *AURKC* mutation analysis was performed because its role in the mitotic process needs to be unambiguously clarified [25].

The real-time PCR (RT-PCR) method was used as in a previous work [14]. Briefly, total RNA was extracted from H295R and SW13 cells and from 66 ACT and 9 NA samples with an RNeasy Mini Kit (SABioscience) according to the manufacturer's protocol, and stored at -80°C . The concentration and integrity of the RNA and cDNA synthesis were checked. *AURKA*, *AURKB*, *AURKC* and *GAPDH* primer sequences were obtained from previous works [22, 23, 26]. Data were obtained as Ct values according to the guidelines and used to determine ΔCt values ($\Delta\text{Ct} = \text{Ct of the target gene minus Ct of the housekeeping gene}$). The $2^{-\Delta\Delta\text{Ct}}$ equation was used to calculate the fold changes in gene expression between two categories of samples (e.g. ACC vs NA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA) and Microsoft Excel software. Cell line data were analyzed using a two-tailed paired Student's t-test and RT-PCR data with the Kruskal-Wallis analysis and Dunn's posttest. The Mann-Whitney test was used for band quantification. Survival analyses were done using a Kaplan-Meier survival plot and two-sided log-rank tests (using a two-fold increase in the median values of normal adrenal tissue gene expression as a cut-off).

A $p < 0.05$ was considered statistically significant. Data are presented as medians and quartiles.

Results

AK inhibitors affect cell viability

MTT assay was performed to examine cell viability in SW13, H295R, primary and metastatic tumor cells. As shown in Fig. 1a, VX-680 induced a concentration- and time-dependent inhibition of mitochondrial activity in the SW13 cells with an IC_{50} of 84 ± 7 nM at 72 h. Treatment of the H295R cells indicated that no IC_{50} was reached (Fig. 1b). The data in Fig. 1c–d show that SNS314 and ZM447439 may reduce SW13 cell viability, especially at 72 h, whereas H295R cells do not respond to the drug treatments (Supplementary Fig. 1A–B). Among the primary adrenocortical tumor cell samples, only APA revealed a slight decrease in cell viability when VX-680 was used at 200 nM (Fig. 1e). In metastatic cell cultures, VX-680 induced a reduction in cell viability, particularly at 200 nM (Fig. 1f).

The VX-680 + mitotane combination was applied to see if the selective pharmacological adrenocortical therapy (mitotane) could enhance the effect of the AKI. VX-680 + mitotane did not induce any marked reduction in cell viability (Supplementary Fig. 1C–D): the CI for VX-680 (200 nM) + mitotane (10 μM) in the SW13 cells at 72 h was 2.1 (the CI could not be calculated at 24 h and for the H295R cells because the IC_{50} could not be reached).

Reversan alone induced a reduction in mitochondrial activity in SW13 cells, particularly at 72 h and at higher concentrations, while no significant changes were seen in the H295R cells (Fig. 2a–b). A combination of reversan 5 μM with VX-680 (at 10 and 200 nM) induced a marked decrease in SW13 cell viability, especially at 72 h, while the H295R cells did not respond to this combination (Fig. 2c–d). Reversan 5 μM was used (in combination with VX-680) to resemble its possible administration in humans, while reversan alone reached an IC_{50} of 95 ± 7 μM at 48 h and 24 ± 6 μM at 72 h.

Proliferation rate modulated by VX-680

$[^3\text{H}]$ thymidine incorporation assays were performed to assess the SW13 and H295R cell proliferation rate. VX-680 reduced the proliferation of SW13 cells, especially at 72 h and 200 nM (Fig. 3a–b). No substantial differences were seen for the H295R cells (Supplementary Fig. 1E–F). Only 1 APA sample showed an evident reduction in proliferation rate at 200 nM (Fig. 3c).

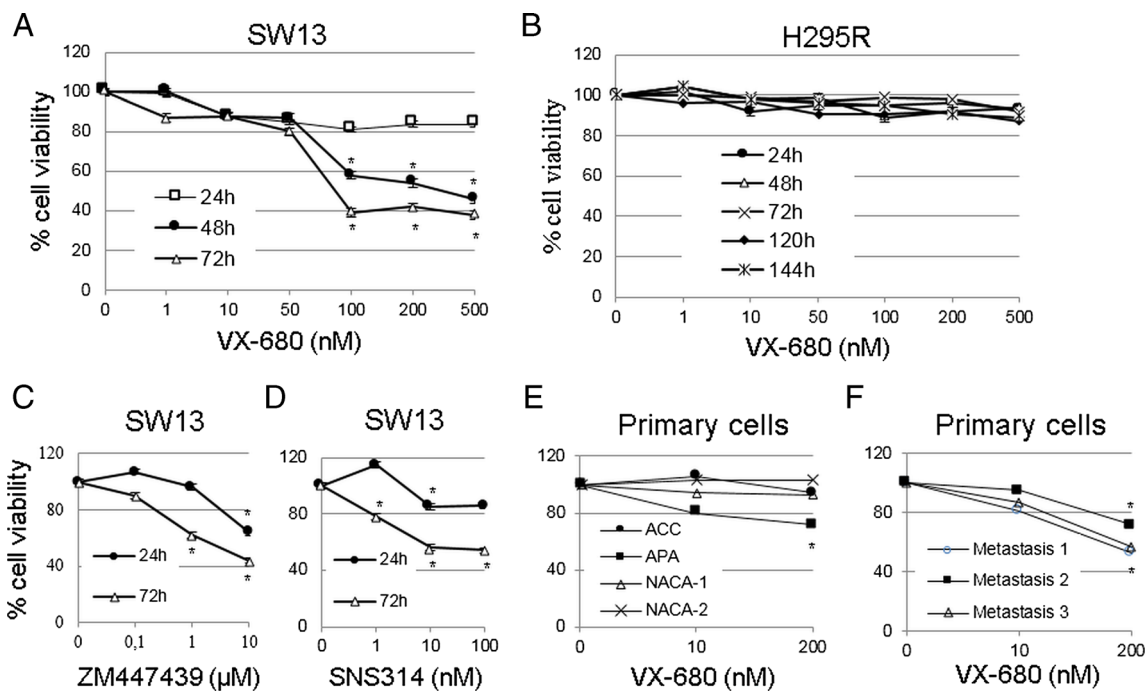


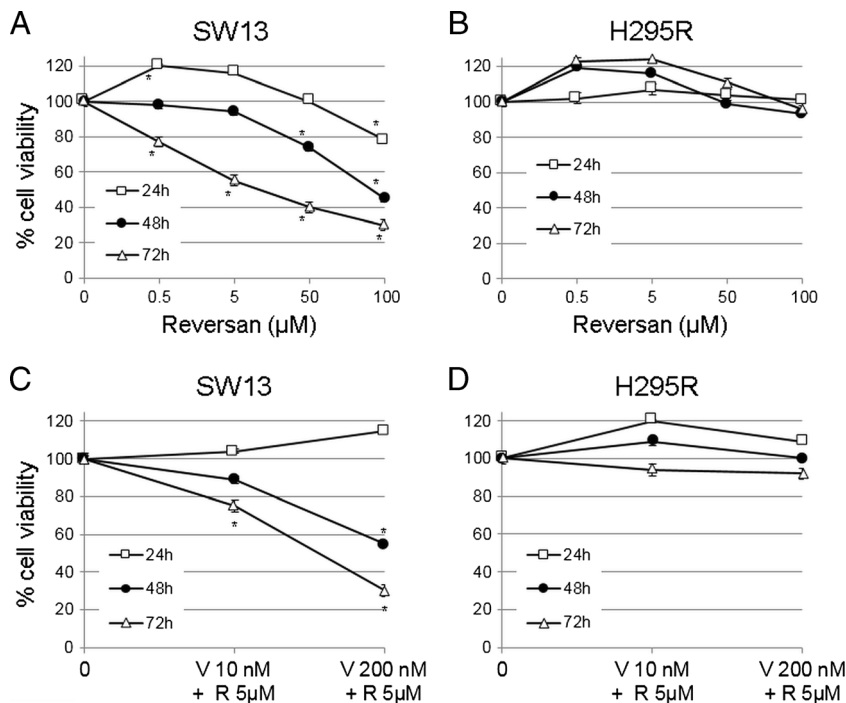
Fig. 1 Cell viability of adrenocortical tumor cells on MTT assay. **a** and **b**: cell viability in SW13 and H295R cells after different times and concentrations treatments. **c** and **d**: cell viability in SW13 cells after 24 h and 72 h of treatment with ZM447439 and SNS314. **e**: 4 primary adrenocortical tumor cell lines (1 ACC, 1 APA and 2 NACA) treated with VX-680 for 24 h. **f**: 3 metastatic tumor cell lines (from lung cancer) treated with VX-680 for 24 h. Treatment vs control: * $p < 0.05$. Experiments performed in quadruplicate and repeated 3 times

Cell morphology changes after VX-680 treatment

Wright’s staining method emphasized sharp morphological changes in the SW13 cells. VX-680 enables the cells to enter

mitosis, but prevents their complete cytokinesis. The cells consequently tend to remain aggregated, forming clusters. Our data showed that the SW13 cells treated with VX-680 at 200 nM were larger than the control and multinucleated

Fig. 2 Cell viability of cell models on MTT assay. **a** and **b**: cell viability in SW13 and H295R cells after different times and concentrations of reversan treatment. **c** and **d**: cell viability in SW13 and H295R cells after 24, 48 and 72 h of treatment with VX-680 (indicated as V) and reversan (indicated as R). Treatment vs control: * $p < 0.05$. Experiments performed in triplicate and repeated 3 times



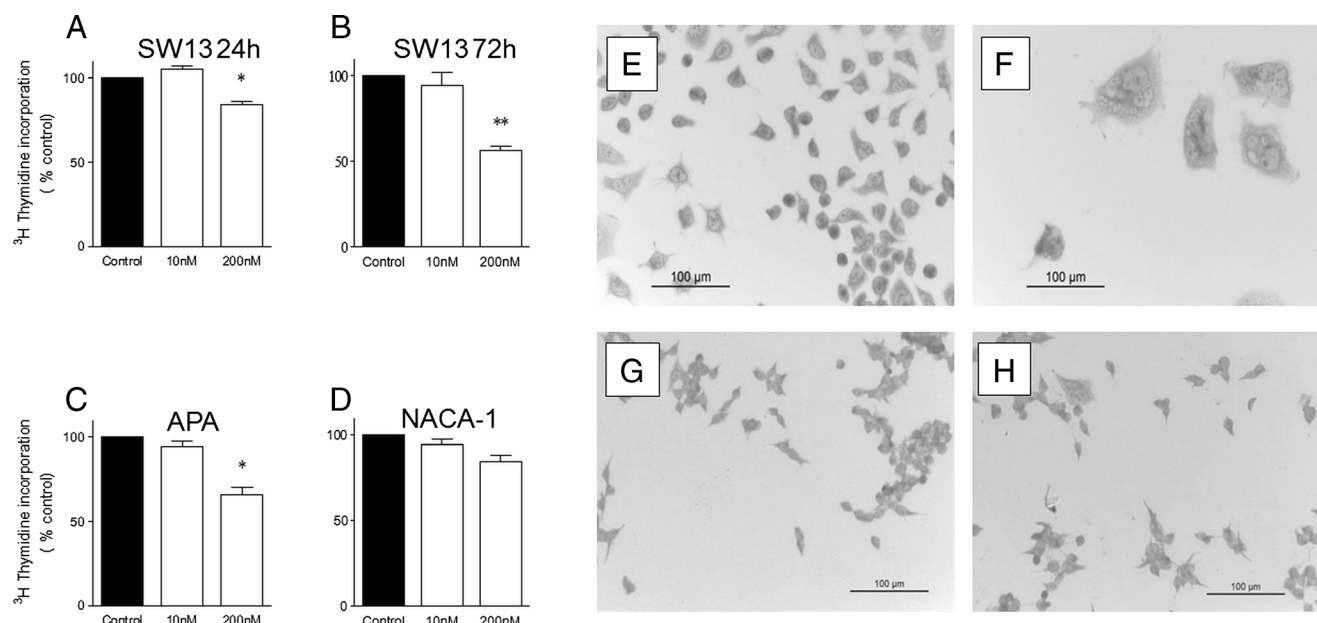


Fig. 3 Adrenocortical cell proliferation and morphological changes. **a, b, c, d:** Proliferation rate of adrenocortical cells treated with VX-680 and assessed by [³H] thymidine incorporation assay. The results are expressed as a percentage of control (100 %). (**a** and **b**: SW13 cells at 24 h and 72 h. **c** and **d**: NACA-1 and NACA-2 cells at 24 h. Treatment vs control: * $p < 0.05$; ** $p < 0.01$. Experiments performed in quadruplicate and

repeated 3 times). **E, F, G, H:** SW13 cell morphology at 72 h and H295R at 144 h, assessed with Wright's staining (**E**: SW13 control cells. **F**: SW13 cells treated with VX-680 at 200 nM. **G**: H295R control cells. **H**: H295R cells treated with VX-680 at 200 nM. Experiments performed twice)

(Fig. 3e–f). Similar results were obtained with higher concentrations of SNS314 and ZM447439 (data not shown). No appreciable changes occurred in the H295R cells (Fig. 3g–h).

Flow cytometry analysis after VX-680 treatment

Based on the role of AK in cell cycle progression, we examined the effect of VX-680 on SW13 and H295R cells. As expected, the drug induced a G2/M phase increase after treatment at 100, 200 and 500 nM in SW13 cells, and a concomitant G1 phase reduction (Fig. 4a). The same could not be said for H295R cells, in which no cell cycle modulation was detected even after prolonged treatment times (Fig. 4b).

To see whether AKI could lead to apoptosis, we analyzed the effect of VX-680 on the fate of apoptotic or necrotic cells. Annexin V-FITC flow cytometry analysis revealed no increase in the cell death process in either SW13 or H295R cell lines (Supplementary Fig. 2A–D).

AK are expressed in SW13 and H295R cells

Western blot analysis confirmed the presence of all antigens tested, and of AK in particular (Fig. 4c). Aurora kinase A was not modulated by the drug treatment, unlike Aurora kinase B and p- Aurora kinases A, B, C (Fig. 4c). Both cell lines showed a decrease in the signal for Aurora kinase B and p- Aurora kinase B (Supplementary Fig. 3A–D).

We also analyzed the MAPK and PI3K/Akt pathways in SW13 and H295R cells because there are reports in the literature of these pathways being modulated by AKI [27, 28]. No substantial change was seen using VX-680, however. Only p-Akt increased slightly in the SW13 cells after treating them with VX-680 at 10 and 200 nM ($p < 0.05$, Supplementary Fig. 3E). We analyzed an initiator caspase (CASP9) and an effector caspase (CASP3) too, but no clear reactivity modulation emerged in either of the cell lines (Fig. 4c).

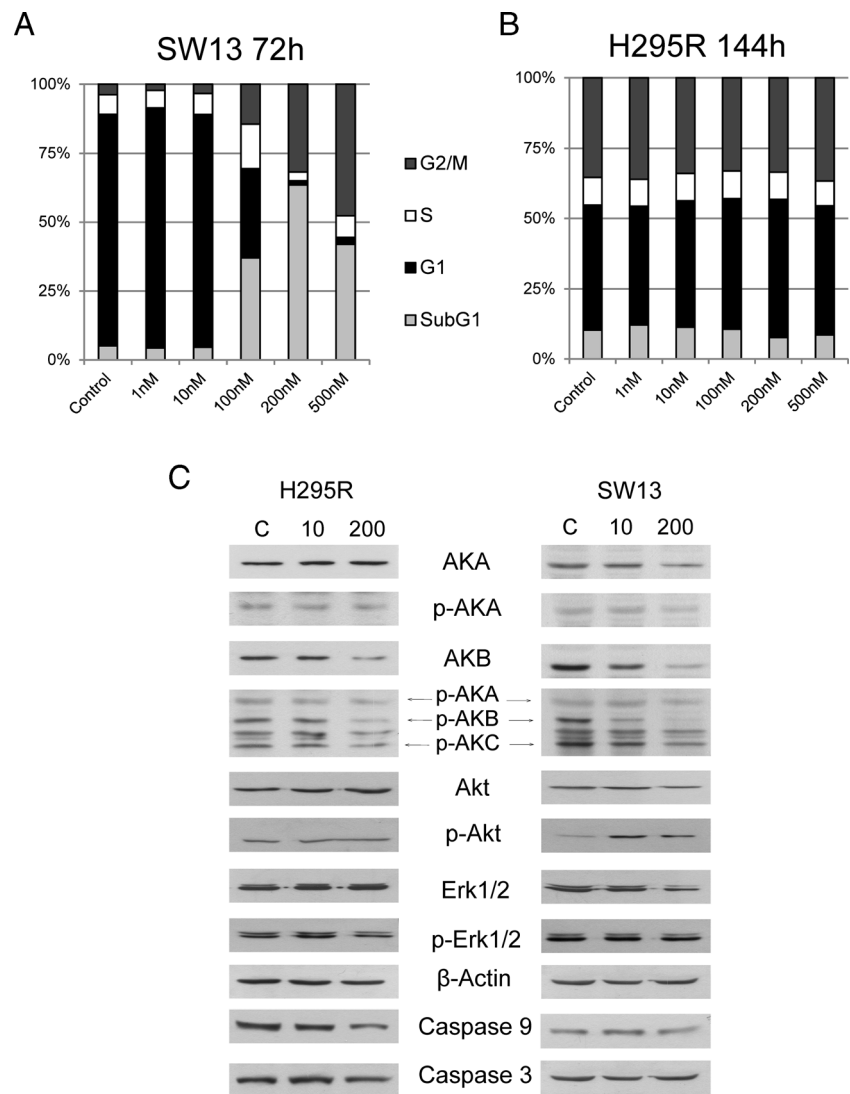
Mutation analysis and gene expression profile

AURKA and *AURKB* gene mutation analysis revealed a similar pattern for the SW13 and H295R cells (Table 2), with one novel alteration (c.42 + 1_42 + 61dup).

Our RT-PCR data confirmed the presence of *AURKA*, *AURKB*, *AURKC* mRNA in both cell lines and in human tissues. *AURKA* and *AURKB* expression was significantly higher in ACC patients ($p < 0.001$) and NACA patients ($p < 0.05$) than in NA, while the difference for APA patients did not reach statistical significance, though there seemed to be the same trend (Fig. 5). No differences in expression were apparent between ACC, NACA and APA for either *AURKA* or *AURKB* (Fig. 5).

As expected, *AURKA* and *AURKB* expression was correlated in ACT patients too ($p < 0.001$), while no such association was found between *AURKA* and *AURKC*, or between *AURKB* and *AURKC*. Due to the small numbers involved in

Fig. 4 Cell cycle distribution and protein alteration in SW13 and H295R cells. **A** and **B**: Representative cell cycle analysis for cell lines treated with VX-680 at different concentrations (**a**: SW13 cells at 72 h. **b**: H295R cells at 144 h. Experiments performed in triplicate). **c**: Representative Western blot at 72 h analyzed for AKA, p-AKA, AKB, p-AKA/AKB/AKC, Akt, p-Akt, Erk1/2, p-Erk1/2, β -actin; C, control (10 = treatment with VX-680 at 10 nM. 200 = treatment with VX-680 at 200 nM; experiments performed in triplicate)



our sample, *AURKC* expression could not be considered as amplified in statistical terms, but a similar trend emerged for this set too (data not shown). H295R and SW13 cells expressed high levels of *AURKA* ($2^{-\Delta\Delta Ct} = 2.5$ and 2.3 , respectively) and *AURKB* ($2^{-\Delta\Delta Ct} = 16.5$ and 15.4 , respectively), but not of *AURKC* ($2^{-\Delta\Delta Ct} = 0.5$ and 0.2 , respectively). Kaplan-Meier survival curve analysis showed no statistical significant difference in ACC event-free survival as far as *AURKA* or *AURKB* gene expression were concerned, though

a trend could be hypothesized (log-rank Mantel-Cox test $p = 0.18$, see Supplementary Fig. 4).

Discussion

Our work demonstrates the efficacy of AKI in adrenocortical tumor in vitro models, confirming the expression of Aurora kinases A, B and C in adult adrenocortical tumor tissues, and

Table 2 Genetic alterations of Aurora kinase A and Aurora kinase B genes

	Aurora kinase A	Aurora kinase B
H295R cells	c. 42 + 1_42 + 61dup (heterozygous) § c. 91 T > A (p. F31I) (heterozygous) c. 169 A > G (p.I57V) (heterozygous)	c. 885C > T (p.S295S) (homozygous)
SW13 cells	c. 42 + 1_42 + 61dup (homozygous) § c. 91 T > A (p. F31I) (homozygous)	c. 885C > T (p.S295S) (homozygous) c. 893 T > C (p.M298 T) (homozygous)

§ Alteration never described in literature, found in 5'-UTR

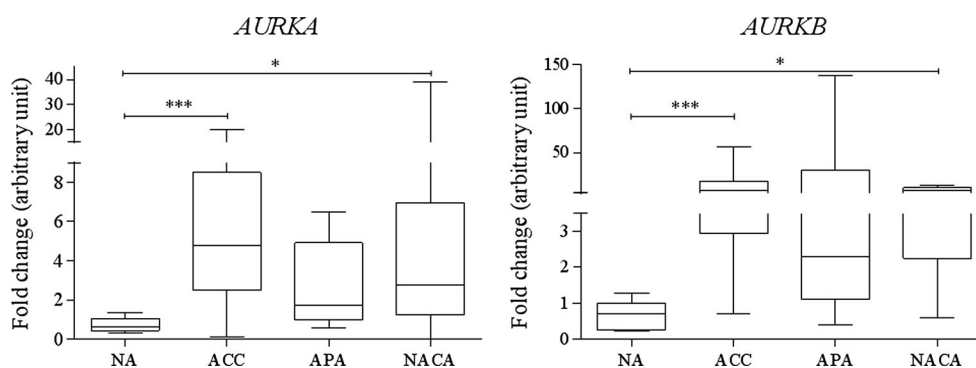


Fig. 5 *Aurora kinase A (AURKA)* and *Aurora kinase B (AURKB)* expression in 66 ACT patients. The graph shows Y-value median-quartile boxplots with decile error bars. NA = normal adrenals,

ACC = adrenocortical carcinomas, APA = aldosterone-producing adenomas, NACA = non-aldosterone-secreting cortical adenomas. * $p < 0.05$; *** $p < 0.001$

in SW13 and H295R cell lines, and showing that AKI are especially effective on primary adrenocortical metastases from NSCLC, and on SW13 cells. The latter are an interesting model of metastasis, the only one available for studying metastatic potential in the adrenal glands. It is worth emphasizing that chemotherapy regimens for treating adrenal metastases (that cannot be resected) could be another fundamental aim in the fight against cancer. A compound such as VX-680, which actively terminates the cells' ability to duplicate may have a second-line role in the treatment of metastases and play an active part in a pharmacological cocktail [29]. VX-680 has never been tested in adrenocortical models. It was recently included in a phase 2 study on different subtypes of leukemia, which goes to show the progress made with AKI in clinical research [30]. Our study is the first in which adrenocortical tumor cells derived from adrenal metastases (of NSCLC) have been treated with VX-680, demonstrating its anti-proliferative effect. In this regard, VX-680 could have potential as a drug with remarkable effects on metastases from NSCLC, though more samples and more research are naturally needed to support this impression.

MTT analysis emphasized the efficacy of the 3 AKI on SW13 cell viability at different times and in various concentrations, with a prevalent effect of VX-680 (Fig. 1a), which convinced us to focus our efforts on this compound. More specifically, VX-680 induced a concentration- and time-dependent inhibition of mitochondrial activity in SW13 cells, while H295R cells seemed to respond differently to AKI, giving the impression of a more cytostatic effect (Fig. 1b and Supplementary Fig. 1A-B). Among the primary adrenocortical tumor cell samples, only APA revealed a slight decrease in cell viability (Fig. 1e). In metastatic cells (Fig. 1f), the effects of VX-680 seemed intense and constant: we surmise that the compound might be very effective on actively proliferating cells (such as metastatic cells), which have a high capacity for replication [31].

Given the paucity of results in H295R cells, we tested the P-gp inhibitor reversan, partly because a recent work

suggested P-gp activation in SW13 and H295R cells, and also because AKI could be a P-gp substrate [7]. The compound was not effective in reversing P-gp-mediated resistance, however, and it had no effect on cell proliferation up to a concentration of 100 μM in H295R cells and 72 h of incubation (Fig. 2b-d). There could be several reasons for the lack of reversan activity in H295R cells: a) P-gp gene (ABCB1) mutations; b) P-gp may be so overexpressed that higher amounts ($>100 \mu\text{M}$) are needed to inhibit its activity; c) other ABC superfamily genes may be involved in MDR; d) other MDR proteins may participate in H295R cell resistance; or e) VX-680 might not be recognized as a substrate by ABCB1, even though this had already been demonstrated elsewhere [32]. Nonetheless, we can exclude *AURKA* and *AURKB* alterations as potential sources of H295R cell resistance, but more efforts are needed to clarify this complex picture. In SW13 cells, on the other hand, we saw a substantial effect of reversan, alone or in combination with VX-680 (Fig. 2a-c). In particular, using VX-680 at 200 nM and reversan at 5 μM may have an additive effect, as demonstrated by MTT assay (Fig. 2c).

^3H thymidine assay generated similar cell proliferation results to MTT analysis, again showing a different behavior of the 2 cell lines (Fig. 3a-b and Supplementary Fig. 1E-F). We undertook a cell morphology analysis in an effort to clarify the effects of AKI in SW13 and H295R cells. We found that SW13 cells treated with VX-680 were aberrantly large, with multiple nuclei, while H295R cells were not (Fig. 3e-h). This may be attributable partly to drug effects (which prevent the completion of cytokinesis), and partly to a different genetic background. In fact, H295R cells carry diverse mutations, such as p.S45P in *CTNNB1* and a homozygous deletion of exons 8-9 of *TP53*, whereas SW13 cells carry no mutations in *CTNNB1* and a homozygous point mutation in exon 6 of *TP53*. A novel alteration was identified as well, i.e. 42 + 1_42 + 61dup (homozygous in SW13 cells and heterozygous in H295R cells) in the 5'-UTR region of the *AURKA* gene. Other known homozygous polymorphisms were found

(Table 2) that might contribute to cell diversity [22, 30]. The meaning of these alterations remains unknown.

One of the main effects of AKI is to generate polyploid cells as a result of multiple rounds of DNA synthesis in the absence of cytokinesis [33]. Cell cycle analysis revealed that the G2/M phase increased in SW13 cells, especially at higher VX-680 concentrations. This finding suggests the presence of endoreduplication, a hallmark of Aurora kinase B inhibition [34, 35], as seen on cell morphology analysis (Fig. 2e-f), and Western blot quantification (Fig. 4c and Supplementary Fig. 4A-D). G2/M arrest may lead to cell death, and an increase in the subG1 cell population in SW13 cells was clearly evident, which might be related to necrosis and apoptosis (Fig. 4a). Flow cytometry analysis with Annexin V-FITC was performed to distinguish between these two different cell fates. This method revealed no substantial changes in either cell line (Supplementary Fig. 2A-B), as confirmed by Western blot, and caspase 3 and 9 were not modulated by VX-680 treatments either (Fig. 4c). We consequently surmise that the cell death observed in SW13 cells on cell cycle analysis (subG1 increased at high drug concentrations) is more likely to be attributable to necrotic processes (toxic effects) than to apoptosis [36]. H295R cells seemed to behave differently: no endoreduplication or polyploid cells emerged on morphological (Fig. 3g-h) or cell cycle analysis (Fig. 4b). This may be because H295R cells are associated with a specific genetic background or already occasionally multinucleated (with the consequences that this implies) [15]. Alternatively, it could be that the integrity of the p53-p21^{Waf1/Cip1} pathway in the G1 postmitotic checkpoint, which seems to govern response to VX-680, may already be impaired [37]. More data are needed to elucidate these results.

Western blot analysis showed that VX-680 inhibited AK expression, particular affecting Aurora kinase B – in fact, the drug is known to have a predilection for targeting Aurora kinase B (Fig. 4c) [35]. This result may be associated with the stronger Aurora kinase B expression emerging from qRT-PCR (Fig. 5), and the more Aurora kinase B is expressed, the more it is inhibited by VX-680. As expected, p-Aurora kinase A, B and C were found decreased, suggesting that VX-680 could take effect on appropriate biochemical targets. In addition, p-Akt was increased in SW13 cells treated with VX-680 at 200 nM, while total Akt expression was normal (Fig. 4c). This finding was unexpected, and no clear link seemed to exist between Akt phosphorylation and AK inhibition. Only one previous work supported this result, and the authors suggested that Aurora kinase A might activate Akt and induce chemoresistance in ovarian cancer cells [38]. Akt activation might be understood in the light of the fact that a blockade on other main signaling pathways (induced by AKI) could reverberate on the Akt pathway, inducing a slight Akt activation. We also analyzed the MAPK and PI3K/Akt pathways in SW13 and H295R cells because other publications

have demonstrated a modulation of these pathways by AKI [27], but no substantial changes came to light using VX-680.

In addition to our Western blot results, qRT-PCR demonstrated *AURKA*, *AURKB*, *AURKC* expression in ACT samples and in 2 cell lines. As expected, *AURKA* and *AURKB* were found overexpressed in ACC and NACA, as happens in other cancer types. These results demonstrate that AK are present in high concentrations in adrenocortical tumors, suggesting an important role for AK in adrenocortical tumorigenesis (even though AK inhibition seemed to have no beneficial effect in a H295R cell model). *AURKA* and *AURKB* overexpression in ACC patients might be related to a worse event-free survival (Supplementary Fig. 4), but it is hard to deduce a trend so it is only by studying a large sample of patients that this hypothesis might be confirmed or rejected.

In conclusion, our novel contribution to clarifying the biochemical features of ACT may pave the way to AKI being used to treat metastatic processes involving adrenal tissue. In particular, VX-680 could be a novel compound for use in association with others in chemotherapies for metastases from NSCLC, or as a second-line clinical approach to metastatic ACC, because this drug acts specifically on the SW13 cell model and metastatic cells. We also demonstrated that the P-gp inhibitor reversan and AKI Vx-680 are of no use in the H295R cell model, and therefore should not be considered when planning primary ACC treatments. Our research on the anti-metastatic potential of AKI is ongoing to extend our human biological series, also to other tumor types.

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Compliance with ethical standards

Disclosure All authors have read the journal's policy on conflicts of interest and have none to disclose.

References

1. Carmena M, Earnshaw WC (2003) The cellular geography of aurora kinases. *Nat Rev Mol Cell Biol* 4:842–854
2. Gautschi O, Heighway J, Mack PC, Purnell PR, Lara PN Jr, Gandara DR (2008) Aurora kinases as anticancer drug targets. *Clin Cancer Res* 14:1639–1648
3. Fu J, Bian M, Jiang Q, Zhang C (2007) Roles of aurora kinases in mitosis and tumorigenesis. *Mol Cancer Res* 5:1–10
4. Borges KS, Moreno DA, Martinelli CE Jr, Antonini SR, de Castro M, Tucci S Jr, Neder L, Ramalho LN, Seidinger AL, Cardinali I, Mastellaro MJ, Yunes JA, Brandalise SR, Tone LG, Scrideli CA

- (2013) Spindle assembly checkpoint gene expression in childhood adrenocortical tumors (ACT): overexpression of aurora kinases a and B is associated with a poor prognosis. *Pediatr Blood Cancer* 60: 1809–1816
5. Obreque-Balboa JE, Sun Q, Bernhardt G, König B, Buschauer A (2016) Flavonoid derivatives as selective ABCC1 modulators: synthesis and functional characterization. *Eur J Med Chem* 109:124–133
 6. Kathawala RJ, Gupta P, Ashby CR Jr, Chen ZS (2015) The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. *Drug Resist Updat* 18:1–17
 7. Gagliano T, Gentilin E, Benfimi K, Di Pasquale C, Tassinari M, Falletta S, Feo C, Tagliati F, Uberti ED, Zatelli MC (2014) Mitotane enhances doxorubicin cytotoxic activity by inhibiting P-gp in human adrenocortical carcinoma cells. *Endocrine* 47:943–951
 8. Nguyen DX, Bos PD, Massague J (2009) Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* 9:274–284
 9. Moreno P, de la Quintana BA, Musholt TJ, Paunovic I, Puccini M, Vidal O, Ortega J, Kraimps JL, Bollo Arocena E, Rodriguez JM, Gonzalez Lopez O, Del Pozo CD, Iacobone M, Veloso E, Del Pino JM, Garcia Sanz I, Scott-Coombes D, Villar-Del-Moral J, Rodriguez JJ, Vazquez Echarri J, Gonzalez Sanchez C, Gutierrez Rodriguez MT, Escorisa I, Nuno Vazquez-Garza J, Tobalina Aguirrezabal E, Martin J, Candel Arenas MF, Lorenz K, Martos JM, Ramia JM (2013) Adrenalectomy for solid tumor metastases: results of a multicenter European study. *Surgery* 154:1215–1222 discussion 1222-1213 <http://endocrinediseases.org/adrenal/metastases.shtml>
 11. Libe R (2015) Adrenocortical carcinoma (ACC): diagnosis, prognosis, and treatment. *Front Cell Dev Biol* 3:45
 12. Dy BM, Wise KB, Richards ML, Young WF Jr, Grant CS, Bible KC, Rosedahl J, Harnsen WS, Farley DR, Thompson GB (2013) Operative intervention for recurrent adrenocortical cancer. *Surgery* 154:1292–1299 discussion 1299
 13. Gaujoux S, Brennan MF (2012) Recommendation for standardized surgical management of primary adrenocortical carcinoma. *Surgery* 152:123–132
 14. Mariniello B, Rosato A, Zuccolotto G, Rubin B, Cicala MV, Finco I, Iacobone M, Frigo AC, Fassina A, Pezzani R, Mantero F (2012) Combination of sorafenib and everolimus impacts therapeutically on adrenocortical tumor models. *Endocr Relat Cancer* 19:527–539
 15. Wang T, Rainey WE (2012) Human adrenocortical carcinoma cell lines. *Mol Cell Endocrinol* 351:58–65
 16. Bertazza L, Barollo S, Radu CM, Cavedon E, Simioni P, Faggian D, Plebani M, Pelizzo MR, Rubin B, Boscaro M, Pezzani R, Mian C (2015) Synergistic antitumor activity of RAF265 and ZSTK474 on human TT medullary thyroid cancer cells. *J Cell Mol Med* 19: 2244–2252
 17. Barollo S, Bertazza L, Baldini E, Ulisse S, Cavedon E, Boscaro M, Pezzani R, Mian C (2014) The combination of RAF265, SB590885, ZSTK474 on thyroid cancer cell lines deeply impact on proliferation and MAPK and PI3K/Akt signaling pathways. *Investig New Drugs* 32:626–635
 18. Pezzani R, Rubin B, Redaelli M, Radu C, Barollo S, Cicala MV, Salva M, Mian C, Mucignat-Caretta C, Simioni P, Iacobone M, Mantero F (2014) The antiproliferative effects of ouabain and everolimus on adrenocortical tumor cells. *Endocr J* 61:41–53
 19. Redaelli M, Mucignat-Caretta C, Isse AA, Gennaro A, Pezzani R, Pasquale R, Pavan V, Crisma M, Ribaldo G, Zagotto G (2015) New naphthoquinone derivatives against glioma cells. *Eur J Med Chem* 96:458–466
 20. Barollo S, Pezzani R, Cristiani A, Redaelli M, Zambonin L, Rubin B, Bertazza L, Zane M, Mucignat-Caretta C, Bulfone A, Pennelli G, Casal Ide E, Pelizzo MR, Mantero F, Moro S, Mian C (2014) Prevalence, tumorigenic role, and biochemical implications of rare BRAF alterations. *Thyroid* 24:809–819
 21. Rubin B, Monticelli H, Redaelli M, Mucignat C, Barollo S, Bertazza L, Mian C, Betterle C, Iacobone M, Fassina A, Boscaro M, Pezzani R, Mantero F (2015) Mitogen-activated protein kinase pathway: genetic analysis of 95 adrenocortical tumors. *Cancer Invest* 33:526–531
 22. Hienonen T, Salovaara R, Mecklin JP, Jarvinen H, Karhu A, Aaltonen LA (2006) Preferential amplification of AURKA 91 a (Ile31) in familial colorectal cancers. *Int J Cancer* 118:505–508
 23. Failes TW, Mitic G, Abdel-Halim H, Po'uha ST, Liu M, Hibbs DE, Kavallaris M (2012) Evolution of resistance to aurora kinase B inhibitors in leukaemia cells. *PLoS One* 7:e30734
 24. Barollo S, Pezzani R, Cristiani A, Bertazza L, Rubin B, Bulfone A, Pelizzo MR, Torresan F, Mantero F, Pennelli G, Moro S, Mian C (2013) Functional significance of the novel H-RAS gene mutation M72I in a patient with medullary thyroid cancer. *Exp Clin Endocrinol Diabetes* 121:546–550
 25. Quartuccio SM, Schindler K (2015) Functions of aurora kinase C in meiosis and cancer. *Front Cell Dev Biol* 3:50
 26. Zekri A, Lesan V, Ghaffari SH, Tabrizi MH, Modarressi MH (2012) Gene amplification and overexpression of aurora-C in breast and prostate cancer cell lines. *Oncol Res* 20:241–250
 27. Wan XB, Long ZJ, Yan M, Xu J, Xia LP, Liu L, Zhao Y, Huang XF, Wang XR, Zhu XF, Hong MH, Liu Q (2008) Inhibition of aurora-a suppresses epithelial-mesenchymal transition and invasion by downregulating MAPK in nasopharyngeal carcinoma cells. *Carcinogenesis* 29:1930–1937
 28. Huang XF, Luo SK, Xu J, Li J, Xu DR, Wang LH, Yan M, Wang XR, Wan XB, Zheng FM, Zeng YX, Liu Q (2008) Aurora kinase inhibitory VX-680 increases Bax/Bcl-2 ratio and induces apoptosis in aurora-A-high acute myeloid leukemia. *Blood* 111:2854–2865
 29. Leong L, Madame Curie Bioscience Database, Landes Bioscience, Austin (TX)
 30. Seymour JF, Kim DW, Rubin E, Haregewoin A, Clark J, Watson P, Hughes T, Dufva I, Jimenez JL, Mahon FX, Rousselot P, Cortes J, Martinelli G, Papayannidis C, Nagler A, Giles FJ (2014) A phase 2 study of MK-0457 in patients with BCR-ABL T315I mutant chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood Cancer J* 4:e238
 31. Wagnerova H, Lazurova I, Felsoci M (2013) Adrenal metastases. *Bratisl Lek Listy* 114:237–240
 32. Tavanti E, Sero V, Vella S, Fanelli M, Michelacci F, Landuzzi L, Magagnoli G, Versteeg R, Picci P, Hattinger CM, Serra M (2013) Preclinical validation of aurora kinase-targeting drugs in osteosarcoma. *Br J Cancer* 109:2607–2618
 33. Baldini E, Arlot-Bonnemains Y, Sorrenti S, Mian C, Pelizzo MR, De Antoni E, Palermo S, Morrone S, Barollo S, Nesca A, Moretti CG, D'Armiento M, Ulisse S (2011) Aurora kinases are expressed in medullary thyroid carcinoma (MTC) and their inhibition suppresses in vitro growth and tumorigenicity of the MTC-derived cell line TT. *BMC Cancer* 11:411
 34. Nair JS, Ho AL, Tse AN, Coward J, Cheema H, Ambrosini G, Keen N, Schwartz GK (2009) Aurora B kinase regulates the postmitotic endoreduplication checkpoint via phosphorylation of the retinoblastoma protein at serine 780. *Mol Biol Cell* 20:2218–2228
 35. Keen N, Taylor S (2004) Aurora-kinase inhibitors as anticancer agents. *Nat Rev Cancer* 4:927–936
 36. Samadi A, Loo P, Mukerji R, O'Donnell G, Tong X, Timmermann BN, Cohen MS (2009) A novel HSP90 modulator with selective activity against thyroid cancers in vitro. *Surgery* 146:1196–1207
 37. 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 Res* 66:7668–7677
 38. Yang H, He L, Kruk P, Nicosia SV, Cheng JQ (2006) Aurora-a induces cell survival and chemoresistance by activation of Akt through a p53-dependent manner in ovarian cancer cells. *Int J Cancer* 119:2304–2312