

AZD1152 negatively affects the growth of anaplastic thyroid carcinoma cells and enhances the effects of oncolytic virus *dl922-947*

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Abstract

Novel therapeutic approaches are required for the treatment of anaplastic thyroid carcinoma (ATC), an incurable disease resistant to current available therapies. Aurora B is an important mitotic kinase involved in chromosome segregation and cytokinesis. It is overexpressed in many cancers including ATC and represents a potential target for chemotherapy. The effects of AZD1152, a specific Aurora B kinase inhibitor, have been evaluated against ATC, showing G₂/M accumulation, polyploidy and subsequent cell death by mitotic catastrophe upon drug treatment. Only three administrations of AZD1152 significantly reduced the growth of ATC tumour xenografts. Oncolytic viruses in association with other forms of treatment have proven highly promising in preclinical and clinical reports. The oncolytic adenovirus *dl922-947* is active against ATC cells, and we have evaluated the effects of the association between AZD1152 and *dl922-947*. In cells treated with virus and drug, we report additive/synergistic killing effects. Interestingly, the phosphorylation of histone H3 (Ser10), the main Aurora B substrate, is inhibited by *dl922-947* in a dose-dependent manner, and completely abolished in association with AZD1152. The combined treatment significantly inhibited the growth of ATC tumour xenografts with respect to single treatments. Our data demonstrate that the Aurora B inhibitor AZD1152, alone or in combination with oncolytic virus *dl922-947*, could represent a novel therapeutic option for the treatment of ATC.

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Introduction

Anaplastic thyroid carcinoma (ATC) is one of the most aggressive human malignancies, responsible for up to 40% of mortality from thyroid cancer. Although multimodality treatments are successfully applied for well-differentiated thyroid carcinomas, ATC survival rates have not been improved for decades: after diagnosis, patients have a median survival time of 4–6 months (Smallridge *et al.* 2009). Development and evaluation of novel therapeutic strategies are, therefore, desperately required.

Several approaches of gene therapy have been studied for ATC, such as differentiating therapy to restore radioiodine uptake, suicide therapy and oncolytic viruses. The latter are viral mutants able to replicate selectively in, and destroy tumour cells. The most common approach to achieve selectivity is the deletion of viral genes whose product is necessary for replication in normal cells, but expendable in cancer cells (Mullen & Tanabe 2002).

dl922-947 is an oncolytic adenovirus, which bears a deletion of 24 bp in E1A-conserved region 2 (CR2)

(Heise et al. 2000). This region normally binds to, and inactivates, host cell Rb, thus causing the release of E2F, followed by S-phase entry and subsequent viral DNA replication. Lacking a functional E1A-CR2 region, *dl922-947* mutant is unable to trigger S-phase entry of quiescent normal cells, but can actively replicate in cells with an aberrant G₁-S checkpoint. This checkpoint is lost in almost all cancer cells, and several *in vitro* and *in vivo* studies demonstrate the efficacy of *dl922-947* in a range of cancer cell lines (Heise et al. 2000). Its killing activity exceeds that of adenovirus 5 wild type (Ad5wt) and of the first generation virus *dl1520* (Heise et al. 2000); a phase I trial of *dl922-947* in women with relapsed ovarian cancer is under way (Baird et al. 2008).

We have shown that *dl922-947* is active against ATC cell lines and tumour xenografts (Libertini et al. 2008). However, data accumulated so far suggest that the oncolytic activity of viruses, although impressive, requires assistance in order to reach full efficacy. Using viruses in association with other forms of treatment has proven highly promising in preclinical and clinical reports. The association of viruses with specific drugs, not only able to directly kill tumour cells but also to increase viral oncolytic activity, would represent a powerful therapeutic tool.

The mitotic serine–threonine kinase Aurora B is a chromosomal passenger protein (Keen & Taylor 2009). Together with INCENP, Borealin and survivin, it exerts essential functions during mitosis, such as modifying histones at the chromatin, correcting misattachments while at the centromere, and regulating cytokinesis at the central spindle and later midbody (Keen & Taylor 2009). Aurora B is overexpressed in several human cancers, and its expression directly correlates with malignancy (Katayama et al. 1999, Araki et al. 2004, Chieffi et al. 2004, 2006, Kurai et al. 2005, Smith et al. 2005, Sorrentino et al. 2005, López-Ríos et al. 2006, Vischioni et al. 2006, Qi et al. 2007, Tanaka et al. 2008). Upon Aurora B depletion or inhibition, the cells with intact checkpoint function arrest with 4N DNA content, while those with compromised p53-dependent pathway undergo endoreduplication and apoptosis (Keen & Taylor 2004).

Cells lacking a p53-mediated post-mitotic checkpoint are highly responsive to Aurora B inhibition, thus suggesting a wide therapeutic window between normal and tumour cells (Keen & Taylor 2004). In human ATC, alterations of the p53 tumour suppressor gene are a constant feature (Smallridge et al. 2009), and we have previously shown that Aurora B is overexpressed in ATC (Sorrentino et al. 2005). All these data indicate

that the treatment of ATC could benefit from the use of an Aurora B inhibitor.

It has been reported that the block in G₂/M phase improves viral entry and replication (Seidman et al. 2001); therefore, we have hypothesized that Aurora B inhibition could enhance the oncolytic effects of *dl922-947* adenovirus. We selected AZD1152 as an Aurora B inhibitor due to its high selectivity for Aurora B and its good solubility, which makes it appropriate for clinical use (Wilkinson et al. 2007). Recent studies showed that AZD1152 significantly reduces tumour growth in a panel of solid human cancer xenograft models (Wilkinson et al. 2007).

In this report, we show that AZD1152 is active against ATC cells, inducing cell death through mitotic catastrophe. We also demonstrate that the drug is able to enhance the anti-neoplastic effects of *dl922-947* oncolytic virus in both *in vitro* and *in vivo* models of ATC. Our data hint towards a mechanism of how AZD1152 and *dl922-947* can synergistically kill ATC cells.

Materials and methods

Cells, adenoviruses and drugs

Human ATC cell lines such as BHT101-5, Cal62, FRO and 8505C have been authenticated as shown previously (Schweppe et al. 2008). All ATC cell lines used have a non-functional *p53* gene: in BHT101-5 cells, a 251 Ile→Thr substitution has been reported, 8505C cells present an Arg→Gly in position 248, Cal62 cells are characterized by A161D mutation while FRO cells are *p53* null (Schweppe et al. 2008).

dl922-947 has a 24-bp deletion in E1A-CR2 (Heise et al. 2000). AdGFP is a non-replicating E1-deleted adenovirus encoding green fluorescent protein (GFP; Libertini et al. 2007). Viral stocks were expanded, purified, stored and quantified as previously reported (Libertini et al. 2007).

AZD1152-HQPA was dissolved in DMSO to a final concentration of 10 mM and stored at –20 °C. AZD1152 was dissolved in 0.3 M Tris–HCl (pH 9) to a final concentration of 10 mg/ml, each mouse received 300 µl of suspension i.p., i.e. 3 mg of prodrug, corresponding to 2.5 mg of active drug.

Viability assay

BHT101-5, Cal62, FRO and 8505C cell lines have been treated with a different range of drug concentrations since their sensitivity to AZD1152 treatment varies. Treated cells were fixed with 50% TCA and stained with 0.4% sulforhodamine B in 1% acetic

acid as already described (Libertini *et al.* 2008). The percentages of surviving cells after treatment were calculated by assuming that the number of surviving untreated cells is 100%.

FACS analysis

PH3 and cell cycle

Cells treated with AZD1152, virus or both were harvested by trypsinization, fixed in 70% cold ethanol and prepared as already described (Esposito *et al.* 2009) using antibodies anti-phospho-histone H3 (Ser10) and anti-histone H3 (Upstate, Biotechnology Inc, Waltham, MA, USA).

AdGFP infection

FRO cells were treated for 24 h with AZD1152 25 nM, washed and then infected with AdGFP (0, 25 or 50 pfu/cell). Forty-eight hours post infection, cells were trypsinized, washed, resuspended in 300 µl of PBS and analysed for the emission in FITC channel.

Samples were acquired with a CYAN flow cytometer (DAKO Corporation, San Jose, CA, USA) and analysed using SUMMIT software.

Micronuclei counting

Cells grown on cover slips and treated as described were fixed 15' in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100 10' and then stained 5' with Hoechst 33258 (1 µg/ml, Sigma–Aldrich). The washes were followed by mounting the cover slips onto glass slides with glycerol:PBS 1:1.

Synchronization

Cells were treated for 12 h with thymidine (2 mM), released for 10 h in fresh media and retreated for further 12 h with thymidine. Cells were washed twice in fresh media, then treated and harvested as indicated.

Antibodies for western blot

Procaspase-3 (sc-56053; 1:100, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), caspase-3 (ab13585, 1:500, Abcam, Cambridge, MA, USA), phospho-histone H3 (Ser10) (06570, 1:500, Upstate Biotechnology Inc, Waltham, MA, USA), histone H3 rabbit polyclonal IgG (31949; 1:1000, Upstate) and actin (sc-10731, 1:500, Santa Cruz) are the antibodies used for western blot.

Tumourigenicity assay, viral replication and distribution

Experiments were performed in 6-week-old female athymic mice (Charles River Laboratories International Inc, MA, USA). Mice were maintained at the Dipartimento di Biologia e Patologia Animal Facility. Animal experiments were conducted in accordance with accepted standards of animal care and in accordance with the Italian regulations for the welfare of animals used in the studies of experimental neoplasia. The study was approved by our institutional committee on animal care.

To evaluate the effects of *dl922-947* in combination with AZD1152, FRO cells (1×10^7) were injected into the right flank of 80 athymic mice. After 40 days, tumour volume was evaluated, and the animals were divided into four groups (20 animals/group) with similar average tumour size. Tumour diameters were measured with callipers, and tumour volumes (*V*) were calculated by the formula of rotational ellipsoid: $V = A \times B^2 / 2$ (*A* is the axial diameter and *B* is the rotational diameter).

Viral replication and distribution were evaluated as previously described (Libertini *et al.* 2008).

Statistical analysis

The analysis of the cell killing effect *in vitro* was made by isobolograms generated to calculate the concentration of each agent killing 50% of cells (EC_{50}) using untreated cells or cells treated with one agent only as controls, as previously described (Cheong *et al.* 2008).

Comparisons among different treatment groups in the experiments *in vivo* were made by the ANOVA method and the Bonferroni *post hoc* test using commercial software (GraphPad Prism 4, GraphPad Software Inc, La Jolla, CA, USA). Differences in the rate of tumour growth in mice were assessed for each time point of the observation period.

Results

AZD1152 treatment induces cell death in ATC cells

Aurora B kinase is overexpressed in ATC, and blocking its expression or activity reduces ATC cell growth (Sorrentino *et al.* 2005). To confirm Aurora B as a therapeutic target, we have evaluated the effects of Aurora B inhibitor AZD1152 on the survival of human ATC cell lines FRO, BHT101-5, 8505C and Cal62.

All cell lines are highly sensitive to AZD1152, with an IC_{50} ranging from 8 to 50 nM for Cal62 and

FRO cells respectively (Fig. 1A). Aurora B inhibition induces endoreduplication thus increasing cell diameter; this could underestimate drug effects when standard proliferation assays are used. Therefore, the anti-proliferative effect of AZD1152 was also determined by cell counting. As shown in Fig. 1B, the inhibitor significantly reduces cell number after 1 day of treatment, further confirming the high efficacy of the drug.

AZD1152 blocks Ser10 H3 phosphorylation and induces mitotic catastrophe

Aurora B phosphorylates histone H3 on serine 10 during mitosis (Prigent & Dimitrov 2003). To confirm AZD1152 effects on Aurora B activity, we treated FRO and BHT101-5 cells for 24 h and performed a FACS analysis by using a specific anti-phospho-Ser10 histone H3 antibody (Fig. 1C).

To analyse the effects of Aurora B inhibition on cell cycle, cells were stained with propidium iodide. Cell cycle profiles were also analysed after 48 h to monitor the effects of a prolonged exposure.

As shown in Fig. 2A and B, a dose- and time-dependent increase in polyploid cells was observed; this increase was paralleled by the reduction in Ser10 H3 phosphorylation levels (Fig. 1C). After 24 h of treatment, a subG₁ fraction and G₂/M accumulation were observed in both cell lines. Immunofluorescence analysis shows that the inhibition of Ser10 H3 phosphorylation caused by AZD1152 treatment does not affect chromosome condensation (Fig. 1D).

The increase of cells with $\geq 4N$ DNA content and a subsequent subG₁ suggest that AZD1152-treated cells die through mitotic catastrophe, a form of programmed cell death resulting from aberrant mitosis. Such mitosis does not produce proper

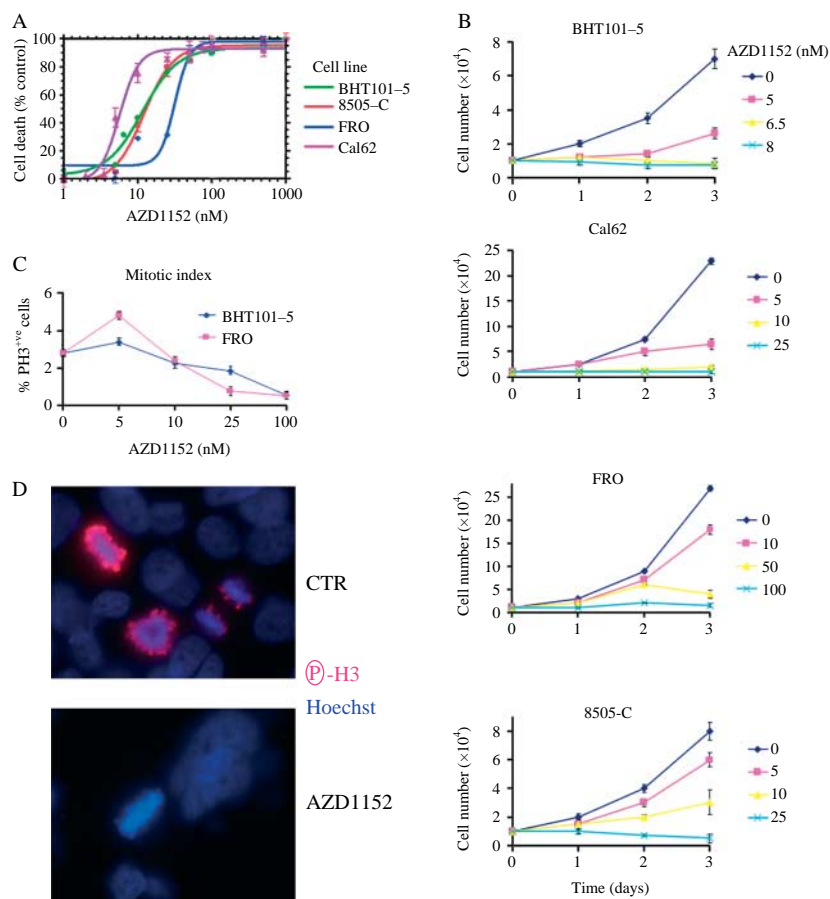


Figure 1 AZD1152 kills ATC cells. (A) ATC cells were treated with AZD1152, and survival was evaluated after 7 days by the sulforhodamine B method. (B) Cells were plated in 12-well plates, treated with different amounts of AZD1152 and counted at the indicated time points. (C) FRO and BHT101-5 cells were treated for 24 h, and phospho-Ser10 H3 levels were evaluated by FACS analysis. The bars in (A), (B) and (C) indicate the s.d. The data are the mean of three different experiments. (D) FRO cells were treated or not for 16 h with 100 nM AZD1152 and phospho-Ser10 H3 visualized by immunofluorescence.

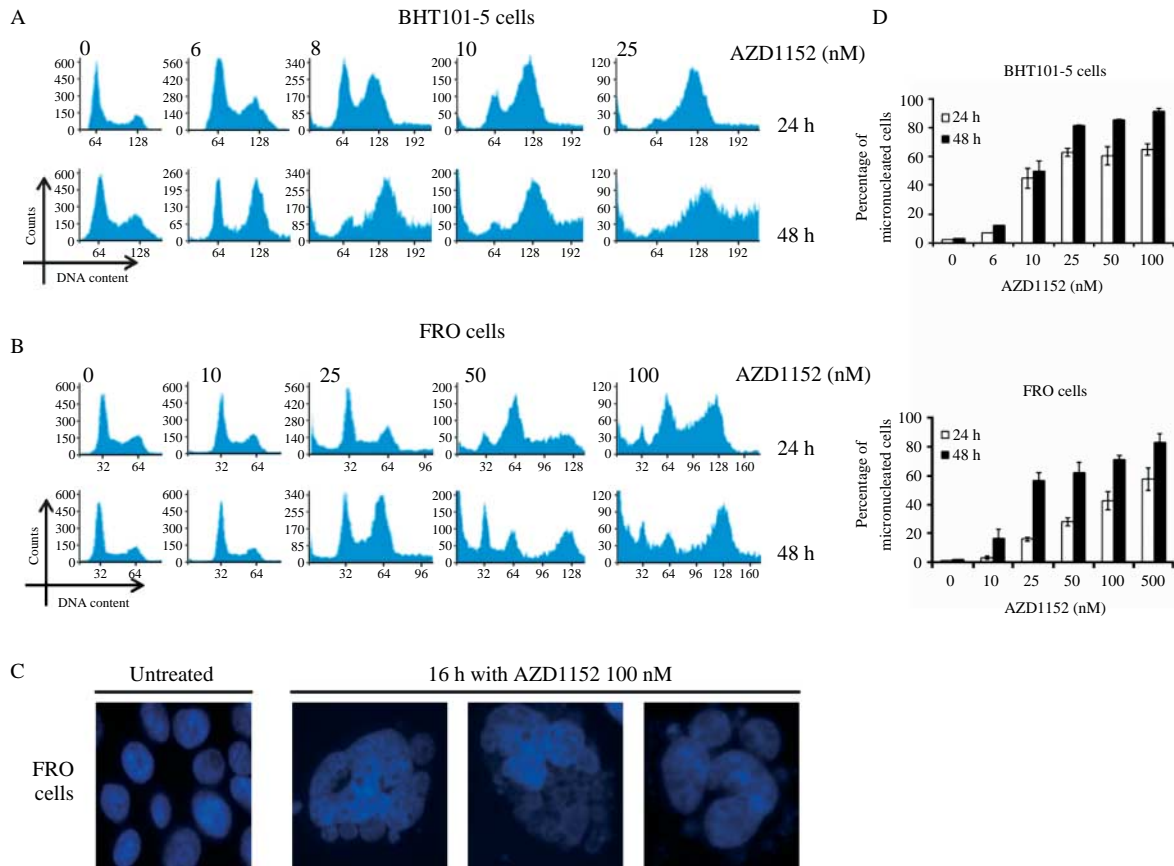


Figure 2 AZD1152 induces mitotic catastrophe. Cell cycle of BHT101-5 (A) and FRO (B) cells was analysed 24 (upper panel) or 48 h (lower panel) after AZD1152 treatment. (C) Hoechst staining of FRO cells treated or not with AZD1152. The same magnification (40 \times) was used in the images. (D) Quantification of data obtained by Hoechst staining. The cells with ≥ 3 micronuclei were counted as positive.

chromosome segregation and cell division, and leads to the formation of large non-viable cells characterized by micronuclei (Castedo *et al.* 2004a). Micronuclei are nuclear envelopes around clusters of missegregated chromosomes (examples in Fig. 2C). By immunofluorescence, we have quantified the number of FRO- and BHT101-5-micronucleated cells after AZD1152 treatment (Fig. 2D). A clear dose- and time-dependent increase in the number of micronucleated cells was observed.

AZD1152 and *dl922-947* show synergistic effects on ATC cells

It has been proposed that drugs interfering with mitosis and cytokinesis could potentiate viral oncolysis (Seidman *et al.* 2001). Therefore, we hypothesized that the association with AZD1152 could positively affect the activity of the oncolytic adenovirus *dl922-947*.

FRO and BHT101-5 cells were treated with AZD1152 and simultaneously infected with *dl922-947*; cell survival was evaluated after 7 days, showing additive/synergistic effects (Fig. 3A, Table 1). In order to understand whether AZD1152 sensitizes cells to viral action, we have treated FRO and BHT101-5 cells with AZD1152 for 24 h and then infected with *dl922-947*. The effects were evaluated after 6 days: the data obtained show that 24 h pre-treatment is sufficient to significantly increase the killing activity of *dl922-947* (Fig. 3B, Table 2), confirming that the inhibitor sensitizes cells to viral action. It is also worth noting that 24 h treatment with AZD1152 alone significantly affects cell survival.

AZD1152 does not affect cellular infectivity

It has been proposed that drugs can enhance viral oncolytic activity by increasing viral entry in target cells (Anders *et al.* 2003). To monitor this step, FRO

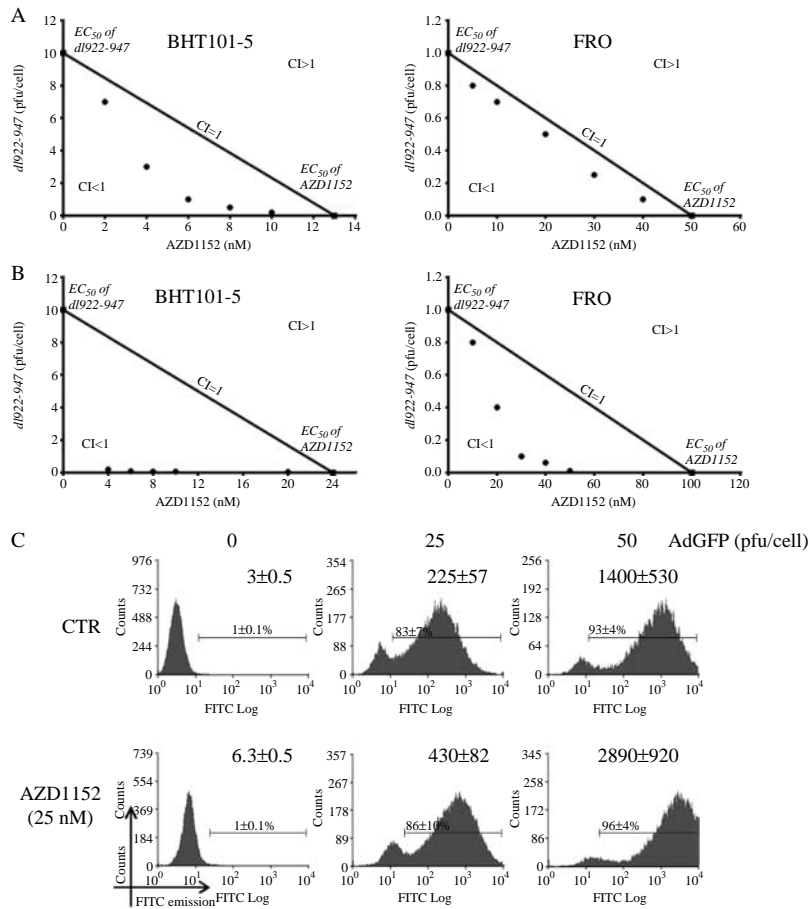


Figure 3 AZD enhances the oncolytic activity of *dl922-947* but does not increase cellular infectivity. (A) FRO and BHT101-5 cells were treated with five fixed combination ratios of *dl922-947* and AZD1152, and cell viability was determined by sulforhodamine B assay 7 days post infection. Dose–response curve and EC₅₀ values were used to construct isobolograms and calculate combination indexes (CI) for each ratio. Ratios are expressed as virus (pfu/cell) to drug (nM). A line is drawn connecting the EC₅₀ values of the virus and drug as a single agent. A ratio producing a CI ≤ 0.8 is considered synergistic, a ratio producing CIs between 0.8 and 1.2 is considered additive and a ratio producing CI ≥ 1.2 is considered antagonistic. (B) Cells were treated for 24 h with the drug; then, the media were replaced with media containing the virus alone. Survival was evaluated after 6 days with isobolograms obtained as above. (C) FRO cells were treated for 24 h with AZD1152, and then infected with AdGFP. Viral entry was quantified by monitoring the percentage of GFP-positive cells (numbers on the bars) and the average expression in FITC channel (numbers on top right) after 24 h. The percentage of GFP-positive cells does not significantly change upon AZD1152 pre-treatment, being ~85% for 25 pfu/cell and ~95% for 50 pfu/cell, regardless of drug pre-treatment. The FITC ratio in infected and uninfected cells upon drug treatment showed no significant change (75 ± 31-fold increase versus 68 ± 19 for 25 pfu/cell and 466 ± 246-fold increase versus 458 ± 182 for 50 pfu/cell).

cells were pre-treated for 24 h with AZD1152 at a low concentration (able to induce G₂/M block but not death after 48 h) and then infected with a non-replicating reporter adenovirus transducing GFP (AdGFP). After additional 24 h, GFP emission was evaluated by cytofluorimetric analysis. The percentage of GFP cells was not modified by AZD1152 pre-treatment, although a positive shift in FITC channel was observed (Fig. 3C). This increase is apparent, since AZD1152 treatment enlarges the cells thus enhancing basal fluorescence. Indeed, analysis of the FITC ratio in infected and uninfected cells treated or not with

AZD1152 showed no significant difference. Similar results were obtained with BHT101-5 cells (data not shown).

***dl922-947* and AZD1152 induce subG₁ phase population in treated cells**

Next, cell cycle profiles in infected cells, treated or not with AZD1152, were evaluated. To better discriminate differences in cell cycle phases, timing and cell death, a double thymidine block was performed to synchronize cells in G₁. As shown in Fig. 4, in untreated cells, 10 h

Table 1 Simultaneous treatment with AZD1152 and *dl922-947*

Cell line	Ratio ((pfu/cell)/(nM))	EC ₅₀	EC ₅₀	CI
		<i>dl922-947</i> (pfu/cell)	AZD1152 (nM)	
BHT101-5	AZD1152 alone	0	13	
	<i>dl922-947</i> alone	10	0	
	1:50	0.2	10	0.79
	1:16	0.5	8	0.67
	1:6	1	6	0.56
	1:1	5	4	0.8
	4:1	8	2	0.95
FRO	AZD1152 alone	0	50	
	<i>dl922-947</i> alone	1	0	
	1:400	0.1	40	0.9
	1:120	0.25	30	0.85
	1:40	0.5	20	0.9
	1:14	0.7	10	0.9
	1:6	0.8	5	0.9

after thymidine release, about 30% of the population re-entered G₁ phase; a similar profile was observed in infected cells. Conversely, after 10 h, AZD1152 induced a G₂/M accumulation that was not modified by the association with *dl922-947*. Starting from 20 h, a significant increase in subG₁ phase was observed in both AZD1152- and *dl922-947*-treated cells. Interestingly, the combination between drug and virus increased the percentage of cells in subG₁ fraction compared with single treatments. It is also worth noting that, in the combined treatment, the increase in subG₁ fraction at later time points is associated with a decrease in polyploidy fractions, suggesting that dying cells (subG₁ fraction) are the ones that escaped G₂/M block.

Caspase-3 activation has been observed in some models of mitotic catastrophe (Castedo *et al.* 2004a,b), and *dl922-947* induces subG₁ accumulation and caspase-3 cleavage in ovarian cancer cells (Baird *et al.* 2008). Therefore, we analysed caspase-3 activation in treated ATC cells. As shown in Fig. 5A, while *dl922-947* induces caspase-3 cleavage, AZD1152 does not. Interestingly, in the combined treatment, an earlier decrease of procaspase-3 with respect to virus treatment alone was observed. This observation suggests that the inhibitor could enhance viral-induced cell death by accelerating the activation of caspase-3 pathway.

***dl922-947* reduces H3 phosphorylation**

FRO cells infected with *dl922-947* show G₂/M accumulation and tetraploidy (Fig. 4), suggestive of a mitotic block. During a normal mitosis, histone H3 is phosphorylated on Ser 10 (28–29); the viral effects on

cell cycle prompted us to analyse whether *dl922-947* acted on this substrate.

We treated FRO cells for 24 h with different amounts of virus and drug, alone or in combination. Cells were stained with propidium iodide and with anti-phospho-Ser10 H3 histone antibody to monitor the percentage of PH3-positive cells during the cell cycle (Fig. 5B). *dl922-947* alone is able to decrease the percentage of PH3-positive cells and, together with AZD1152, Ser10 H3 phosphorylation is almost completely abolished: FACS data were confirmed by western blot analysis of PH3 levels (Fig. 5C).

AZD1152 increases *dl922-947* oncolytic activity *in vivo*

To further validate the potential therapeutic use of AZD1152 in association with *dl922-947*, we analysed the effects of the combined treatment on xenograft tumours. To study the effect of the association in the worst possible scenario, FRO cells were chosen, being the least sensitive to AZD1152 effect (Fig. 1) and viral infection (Libertini *et al.* 2008). A low viral dose (1×10^6 pfu) was used to better evaluate the effects of the combined treatment; virus was administered by i.t. injection to avoid first pass effect.

Animals were divided into four groups: untreated, treated with AZD1152, *dl922-947* or both. As shown in Fig. 6A, AZD1152 and *dl922-947* in combination have a stronger anti-tumour activity than when used alone. As already described, no toxicities were observed in virus-treated animals (Libertini *et al.* 2008). Drug-treated animals showed slight weight loss and dehydration on the third day of treatment, but

Table 2 AZD1152 pre-treatment on *dl922-947*-infected cells

Cell line	Ratio ((pfu/cell)/(nM))	EC ₅₀	EC ₅₀	CI
		<i>dl922-947</i> (pfu/cell)	AZD1152 (nM)	
BHT101-5	AZD1152 alone	0	24	
	<i>dl922-947</i> alone	10	0	
	1:500	0.04	20	0.84
	1:167	0.06	10	0.42
	1:114	0.07	8	0.34
	1:62	0.08	5	0.21
	1:20	0.2	4	0.19
FRO	AZD1152 alone	0	100	
	<i>dl922-947</i> alone	1	0	
	1:5000	0.01	50	0.51
	1:667	0.06	40	0.46
	1:300	0.1	30	0.4
	1:50	0.4	20	0.6
	1:12	0.8	10	0.9

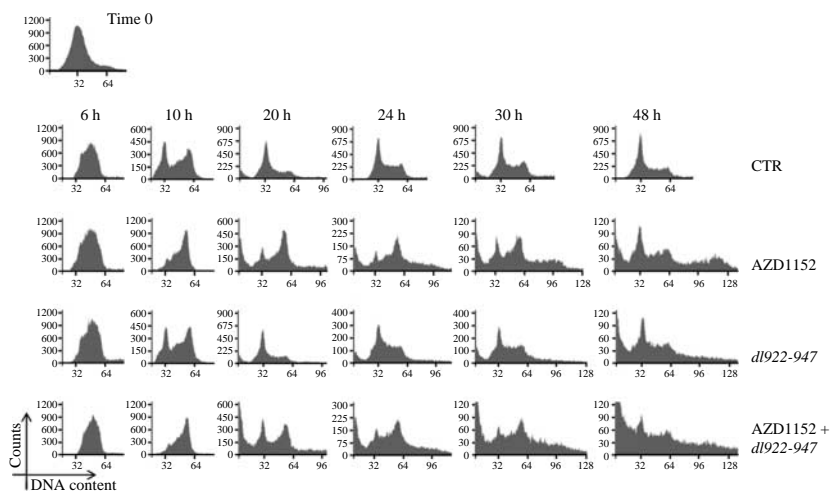


Figure 4 AZD1152 and *dl922-947* induce tetraploidy and cell death. FRO cells were synchronized in G₁ phase and treated with AZD1152 10 nM, *dl922-947* (25 pfu/cell) or both. FACS analysis was performed to analyse the cell cycle.

recovered after 2–3 days. In the combined treatment group, animals took longer to recover (4–5 days), but no other symptoms were observed.

Viral replication analysis in animals pre-treated with AZD1152 showed an increase of genome copies (Fig. 6B). However, this effect was not observed *in vitro* (data not shown). It has been hypothesized that the pre-treatment with anti-neoplastic drugs could improve intratumoural viral diffusion by reducing the number of neoplastic cells (Vähä-Koskela *et al.* 2007). To address this point, FRO xenografts animals, pre-treated or not with AZD1152, were injected intratumourally with AdGFP. GFP expression was evaluated by fluorescence microscopy analysis (Fig. 6C). In control tumours, a faint fluorescence signal was observed, whereas in AZD1152-treated tumours, a more intense fluorescence signal was

detected. It is worth noting that both viral replication and distribution were increased three times upon drug treatment (Fig. 6B and C).

Discussion

ATC is one of the most lethal neoplasia and leads to death in a short time. Active therapies are not available, making the development of novel therapeutic strategies imperative (Smallridge *et al.* 2009).

AZD1152 is a reversible ATP-competitive Aurora inhibitor, which is 1000-fold more selective for Aurora kinase B than for Aurora kinase A (Wilkinson *et al.* 2007). The effects of AZD1152 have been already assessed in *in vivo* models of human leukaemia (Wilkinson *et al.* 2007, Yang *et al.* 2007, Walsby *et al.* 2008, Oke *et al.* 2009), breast cancer (Gully *et al.* 2010),

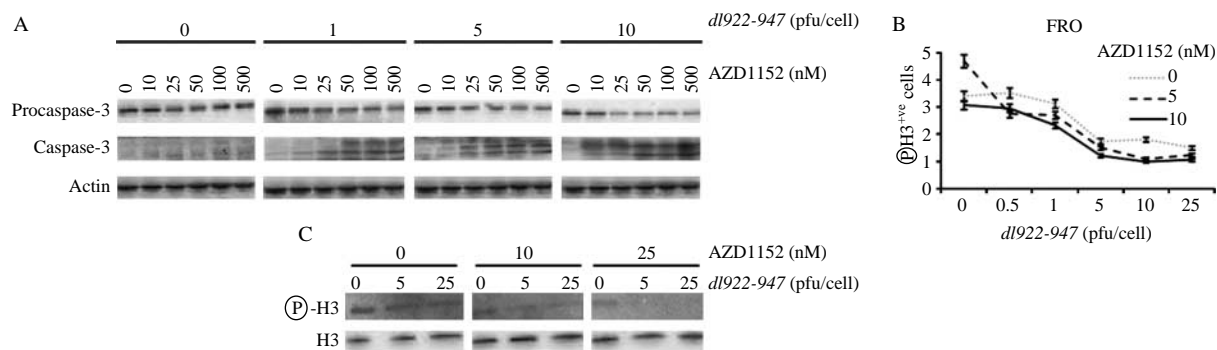


Figure 5 *dl922-947* induces caspase-3 activation and decreases PH3 levels. (A) FRO cells were synchronized in G₁ phase and treated with different combinations of *dl922-947* and AZD1152. Attached and detached cells were harvested, and western blot analysis was performed to quantify procaspase- and activated caspase-3 levels. Fifty micrograms of lysate were loaded in each well. FRO cells were treated for 24 h with different amounts of AZD1152 and *dl922-947*, alone or in combination. Ser10 PH3 levels were evaluated by FACS (B) and western blot analysis (C).

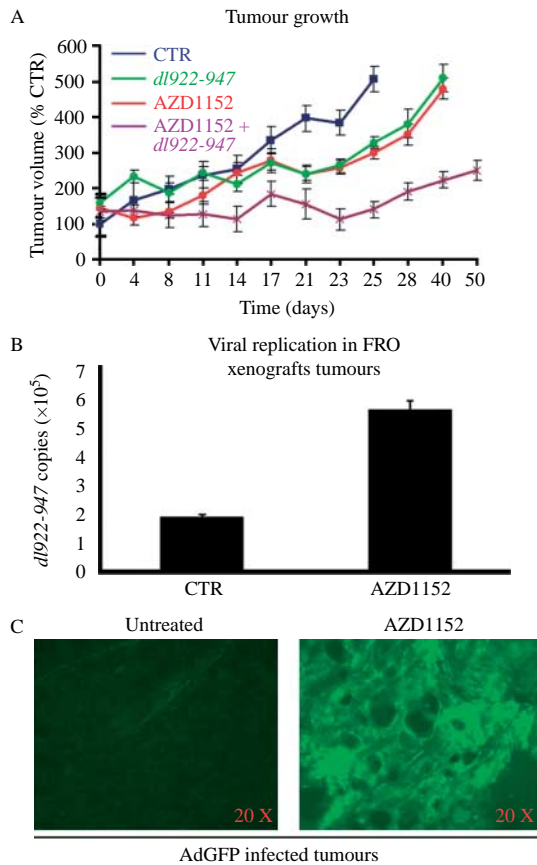


Figure 6 AZD1152 delays tumour growth and facilitates *dl922-947* replication and distribution. (A) Tumour bearing mice were randomized into four groups. Two groups received AZD1152 (100 mg/kg per day) i.p. from T1 to T3 and from T15 to T17. *dl922-947* (1×10^6 pfu, in a volume of 300 μ l) was injected at T4, T8, T11 and T15 into one AZD1152-treated group and one untreated group. The control group was injected with saline solution. Tumour volume is expressed as a percentage of the volume observed at day 0 in the control group. The difference between treated and untreated groups becomes statistically significant ($P < 0.05$) from day 17 for virus versus control and drug versus control, and from day 8 for combined treatment versus control. At day 21, a significant difference ($P < 0.05$) was observed between the combined treatment group and single treatment groups. From day 23 till the end of the treatment, the difference became highly significant ($P < 0.01$). (B) FRO cells (1×10^7 cells) were injected intratumourally within ten athymic mice. Forty days later, when tumours were detectable, animals were divided into two groups, which received respectively AZD1152 (2.5 mg/mouse) or saline solution i.p. After 24 h, *dl922-947* (1×10^6 pfu, in a volume of 300 μ l) was injected intratumourally in both groups. Two days later, animals were killed. DNA was extracted from 100 mg of tumour tissue, and the number of viral copies was evaluated by real-time PCR. The data are the mean of three different experiments. (C) Ten FRO-induced xenograft tumours were injected i.p. with AZD1152 (100 mg/kg per day) or saline solution for 3 consecutive days and, after 2 days, AdGFP (1×10^7 pfu, in a volume of 300 μ l) was injected intratumourally in both groups. Two days later, animals were killed, tumours were excised and GFP distribution was evaluated by confocal microscopy. The quantification of the digitized signal showed a threefold increase upon AZD1152 treatment.

hepatocellular carcinoma (Aihara *et al.* 2010), and colon and lung cancers (Wilkinson *et al.* 2007). AZD1152 and other Aurora inhibitors are currently in early clinical evaluation, showing reversible neutropenia as a major side effect (Keen & Taylor 2009).

In the present study, we have demonstrated that AZD1152 is active against ATC cells and that the effects are observed within 24 h. These data are consistent with the high levels of Aurora B expression (Sorrentino *et al.* 2005), the lack of a functional p53 pathway (Schweppe *et al.* 2008) and the short doubling time (10 h) of ATC cells. In the present study, we have also shown that just three doses of AZD1152 significantly delay the growth of ATC tumour xenografts.

Another Aurora inhibitor, VX-680, has been previously evaluated on ATC cell lines showing inhibition of cell proliferation and cell death; however, the drug was not evaluated *in vivo* (Arlot-Bonnemains *et al.* 2008). Cells treated with VX-680 showed an increase in DNA content ($>4N$), reduction of Ser10 H3 phosphorylation, subG₁ accumulation and activation of caspase-3 (Arlot-Bonnemains *et al.* 2008). The authors concluded that VX-680 activated the apoptotic cascade in ATC cells, and suggested that the effects reported were due to the inhibition of Aurora A rather than Aurora B. VX-680 inhibits Aurora kinases with comparable inhibition constants, ranging from 0.6 nM for Aurora A to 18 nM for Aurora B; hence, it is plausible that the effects exerted by VX-680 on ATC cells are due to the inhibition of both Aurora kinases. In addition, it has been previously reported in other cell models that effects seen with VX-680 closely resemble those described for Aurora B inhibition (Keen & Taylor 2004) and mirror the effects described here with AZD1152 on ATC cells. Moreover, the IC₅₀ of VX-680 on ATC cells ranged from 25 to 150 nM: these concentrations are also sufficient to inhibit the kinase FLT3 (Harrington *et al.* 2004, Arlot-Bonnemains *et al.* 2008). On the other hand, the IC₅₀ of AZD1152 in this study ranges from 5 to 30 nM, indicating that AZD1152 could achieve a therapeutic effect at lower and less toxic concentrations. Therefore, our results clearly indicate using both *in vitro* and *in vivo* models that selective inhibition of Aurora B could represent a therapeutic option for the treatment of ATC.

Oncolytic viruses are emerging as new therapeutic tools for the treatment of cancer, and we previously demonstrated that the mutants *dl1520* and *dl922-947* are active against ATC *in vitro* and *in vivo* (Libertini *et al.* 2007, 2008). It has been reported that drugs able to block cells in G₂/M or inhibit cytokinesis could enhance the effects of oncolytic viruses

(Seidman *et al.* 2001). Since AZD1152 has a clear effect on cell division, we wondered whether it could positively affect *dl922-947* activity. The data presented in this manuscript demonstrate the efficacy of this combination both *in vitro* and *in vivo*.

To understand how AZD1152 enhances oncolytic activity, we first monitored viral entry upon drug treatment. Our data show that AZD1152 does not affect viral entry. The synchronization in G₂/M phase leads to modest, but significant, translocation of CAR receptor to the cell surface of A549 lung carcinoma cells (Seidman *et al.* 2001). Although AZD1152-treated cells accumulate in G₂/M phase, membrane levels of CAR and its co-receptors integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_1$ do not increase after AZD1152 treatment (data not shown). By using AdGFP, we did not observe any difference in viral entry during each cell cycle phase (data not shown). Our observation is not in agreement with Seidman *et al.* (2001); this discrepancy could be due to the different cellular system used or to a very slight increase in receptor levels hidden by the increase in cellular size.

Secondly, we analysed cell death mechanisms induced by virus and drug, alone or in combination. AZD1152 induces a G₂/M block and tetraploidy in ATC cells. This aberrant mitotic phenotype, the presence of micronuclei and the appearance of subG₁ population demonstrate that AZD1152-treated cells die through mitotic catastrophe. According to the literature, mitotic catastrophe could share biochemical hallmarks with apoptosis, such as mitochondrial membrane permeabilization (MMP) and caspase cleavage (Castedo *et al.* 2004a,b). In our study, despite the clear induction of mitotic catastrophe, AZD1152 did not induce caspase-3 activation (Fig. 5) and MMP (data not shown). Like AZD1152-treated cells, ATC-infected cells show enlargement and polyploidy. However, it is possible to exclude death by mitotic catastrophe since, upon infection, cells detach, a feature not observed in mitotic catastrophe. Moreover, in the few adhering cells, we have not observed the presence of micronuclei (data not shown). We have also excluded autophagic cell death, since neither beclin 1 activation nor LC3I-II conversion were induced, and accumulation of autophagic vesicular organelles was not observed by FACS analysis in acridine orange-stained cells (data not shown). Our data extend to ATC cells the observation of Baird *et al.* (2008) that infection with mutant oncolytic adenovirus leads to programmed cell death lacking the features of classical apoptosis, but showing some apoptotic markers, such as subG₁ accumulation and caspase-3 activation. Interestingly, the association with

AZD1152 accelerates the appearance of cleaved caspase-3 and increases the percentage of cells in subG₁ phase. Our results indicate that AZD1152 enhances the cytotoxic effects of oncolytic viruses, although the cell death mechanisms are not yet defined.

During mitosis, Aurora B is involved in phosphorylation of histone H3 on Ser10 (Ditchfield *et al.* 2003, Hauf *et al.* 2003). Accordingly, AZD1152 strongly reduces H3 phosphorylation. It has been reported that the oncolytic herpes virus G47D decreases phospho-Ser10 H3 levels (Passer *et al.* 2009), and we observed that the oncolytic adenovirus *dl922-947* (Fig. 5B) and Ad5wt (personal observation) have a similar effect. These data suggest that modulation of PH3 levels is a common feature of viral infections; it is conceivable that viruses prematurely end mitosis in order to switch cell machinery to viral replication.

The first oncolytic adenovirus described, *dl1520* (Heise *et al.* 1997), has shown improved effects in clinical trials in combinations with chemotherapy (Kumar *et al.* 2008). In 2005, China approved the world's first oncolytic virus therapy for cancer treatment, with modified adenovirus H101, similar to *dl1520* (Yu & Fang 2007). In a phase III trial, a 79% response rate for H101 plus chemotherapy, compared with 40% for chemotherapy alone, was observed (Yu & Fang 2007). Other oncolytic viruses have entered into clinical trials: their efficacy and safety, and synergistic effects in association with other drugs have been demonstrated, confirming virotherapy as a promising direction for the treatment of cancer (Liu *et al.* 2007). Oncolytic viruses target neoplastic cells using a mechanism different from that of anti-neoplastic drug; therefore, the combination treatment could contribute to avoid the development of resistant cancer cells, thus increasing the cure rate.

Data presented here demonstrate that the selective inhibitor of Aurora B kinase AZD1152 is highly effective against ATC cell lines and tumour xenografts, and could represent a novel therapeutic option for the treatment of this dismal disease. We have also shown that AZD1152 enhances the effects of *dl922-947* against ATC; similar combined approaches could be used for the treatment of ATC and other aggressive and incurable human cancers.

Declaration of interest

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

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References

- Aihara A, Tanaka S, Yasen M, Matsumura S, Mitsunori Y, Murakata A, Noguchi N, Kudo A, Nakamura N, Ito K *et al.* 2010 The selective Aurora B kinase inhibitor AZD1152 as a novel treatment for hepatocellular carcinoma. *Journal of Hepatology* **52** 63–71. (doi:10.1016/j.jhep.2009.10.013)
- Anders M, Christian C, McMahon M, McCormick F & Korn WM 2003 Inhibition of the Raf/MEK/ERK pathway up-regulates expression of the coxsackievirus and adenovirus receptor in cancer cells. *Cancer Research* **63** 2088–2095.
- Araki K, Nozaki K, Ueba T, Tatsuka M & Hashimoto N 2004 High expression of Aurora-B/Aurora and Ippll-like midbody-associated protein (AIM-1) in astrocytomas. *Journal of Neuro-Oncology* **67** 53–64. (doi:10.1023/B:NEON.0000021784.33421.05)
- Arlot-Bonnemains Y, Baldini E, Martin B, Delcros JG, Toller M, Curcio F, Ambesi-Impiombato FS, D'Armiento M & Ulisse S 2008 Effects of the Aurora kinase inhibitor VX-680 on anaplastic thyroid cancer-derived cell lines. *Endocrine-Related Cancer* **15** 559–568. (doi:10.1677/ERC-08-0021)
- Baird SK, Aerts JL, Eddaoudi A, Lockley M, Lemoine NR & McNeish IA 2008 Oncolytic adenoviral mutants induce a novel mode of programmed cell death in ovarian cancer. *Oncogene* **27** 3081–3090. (doi:10.1038/sj.onc.1210977)
- Castedo M, Perfettini JL, Roumier T, Valent A, Raslova H, Yakushijin K, Horne D, Feunteun J, Lenoir G, Medema R *et al.* 2004a Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy. *Oncogene* **23** 4362–4370. (doi:10.1038/sj.onc.1207572)
- Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R & Kroemer G 2004b Cell death by mitotic catastrophe: a molecular definition. *Oncogene* **23** 2825–2837. (doi:10.1038/sj.onc.1207528)
- Cheong SC, Wang Y, Meng JH, Hill R, Sweeney K, Kim D, Lemoine NR & Halldén G 2008 E1A-expressing adenoviral E3B mutants act synergistically with chemotherapeutics in immunocompetent tumor models. *Cancer Gene Therapy* **15** 40–50. (doi:10.1038/sj.cgt.7701099)
- Chieffi P, Troncone G, Caleo A, Libertini S, Linardopoulos S, Tramontano D & Portella G 2004 Aurora B expression in normal testis and seminomas. *Journal of Endocrinology* **181** 263–270. (doi:10.1677/joe.0.1810263)
- Chieffi P, Cozzolino L, Kisslinger A, Libertini S, Staibano S, Mansueto G, De Rosa G, Villacci A, Vitale M, Linardopoulos S *et al.* 2006 Aurora B expression directly correlates with prostate cancer malignancy and influence prostate cell proliferation. *Prostate* **66** 326–333. (doi:10.1002/pros.20345)
- Ditchfield C, Johnson VL, Tighe A, Ellston R, Haworth C, Johnson T, Mortlock A, Keen N & Taylor SS 2003 Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. *Journal of Cell Biology* **161** 267–280. (doi:10.1083/jcb.200208091)
- Espósito F, Libertini S, Franco R, Abagnale A, Marra L, Portella G & Chieffi P 2009 Aurora B expression in post-puberal testicular germ cell tumours. *Journal of Cellular Physiology* **221** 435–439. (doi:10.1002/jcp.21875)
- Gully CP, Zhang F, Chen J, Yeung JA, Velazquez-Torres G, Wang E, Yeung SC & Lee MH 2010 Antineoplastic effects of an Aurora B kinase inhibitor in breast cancer. *Molecular Cancer* **9** 42. (doi:10.1186/1476-4598-9-42)
- Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T, Graham JA, Demur C, Hercend T, Diu-Hercend A *et al.* 2004 VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth *in vivo*. *Nature Medicine* **10** 262–267. (doi:10.1038/nm1003)
- Hauf S, Cole RW, LaTerra S, Zimmer C, Schnapp G, Walter R, Heckel A, van Meel J, Rieder CL & Peters JM 2003 The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *Journal of Cell Biology* **161** 281–294. (doi:10.1083/jcb.200208092)
- Heise C, Sampson-Johannes A, Williams A, McCormick F, Von Hoff DD & Kirn DH 1997 ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nature Medicine* **3** 639–645. (doi:10.1038/nm0697-639)
- Heise C, Hermiston T, Johnson L, Brooks G, Sampson-Johannes A, Williams A, Hawkins L & Kirn D 2000 An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nature Medicine* **6** 1134–1139. (doi:10.1038/80474)
- Katayama H, Ota T, Jisaki F, Ueda Y, Tanaka T, Odashima S, Suzuki F, Terada Y & Tatsuka M 1999 Mitotic kinase expression and colorectal cancer progression. *Journal of the National Cancer Institute* **91** 1160–1162. (doi:10.1093/jnci/91.13.1160)
- Keen N & Taylor S 2004 Aurora-kinase inhibitors as anticancer agents. *Nature Reviews. Cancer* **4** 927–936. (doi:10.1038/nrc1502)

- Keen N & Taylor S 2009 Mitotic drivers – inhibitors of the Aurora B kinase. *Cancer Metastasis Reviews* **28** 185–195. (doi:10.1007/s10555-009-9184-9)
- Kumar S, Gao L, Yeagy B & Reid T 2008 Virus combinations and chemotherapy for the treatment of human cancers. *Current Opinion in Molecular Therapeutics* **10** 371–379.
- Kurai M, Shiozawa T, Shih HC, Miyamoto T, Feng YZ, Kashima H, Suzuki A & Konishi I 2005 Expression of Aurora kinases A and B in normal, hyperplastic, and malignant human endometrium: Aurora B as a predictor for poor prognosis in endometrial carcinoma. *Human Pathology* **36** 1281–1288. (doi:10.1016/j.humpath.2005.09.014)
- Libertini S, Iacuzzo I, Ferraro A, Vitale M, Bifulco M, Fusco A & Portella G 2007 Lovastatin enhances the replication of the oncolytic adenovirus dl1520 and its antineoplastic activity against anaplastic thyroid carcinoma cells. *Endocrinology* **148** 5186–5194. (doi:10.1210/en.2007-0752)
- Libertini S, Iacuzzo I, Perruolo G, Scala S, Ieranò C, Franco R, Hallden G & Portella G 2008 Bevacizumab increases viral distribution in human anaplastic thyroid carcinoma xenografts and enhances the effects of E1A-defective adenovirus dl922-947. *Clinical Cancer Research* **14** 6505–6514. (doi:10.1158/1078-0432.CCR-08-0200)
- Liu TC, Galanis E & Kim D 2007 Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress. *Nature Clinical Practice. Oncology* **4** 101–117. (doi:10.1038/npcnc0736)
- López-Ríos F, Chuai S, Flores R, Shimizu S, Ohno T, Wakahara K, Illei PB, Hussain S, Krug L, Zakowski MF et al. 2006 Global gene expression profiling of pleural mesotheliomas: overexpression of aurora kinases and P16/CDKN2A deletion as prognostic factors and critical evaluation of microarray-based prognostic prediction. *Cancer Research* **66** 2970–2979. (doi:10.1158/0008-5472.CAN-05-3907)
- Mullen JT & Tanabe KK 2002 Viral oncolysis. *Oncologist* **7** 106–119. (doi:10.1634/theoncologist.7-2-106)
- Oke A, Pearce D, Wilkinson RW, Crafter C, Odedra R, Cavenagh J, Fitzgibbon J, Lister AT, Joel S & Bonnet D 2009 AZD1152 rapidly and negatively affects the growth and survival of human acute myeloid leukemia cells *in vitro* and *in vivo*. *Cancer Research* **69** 4150–4158. (doi:10.1158/0008-5472.CAN-08-3203)
- Passer BJ, Castelo-Branco P, Buhman JS, Varghese S, Rabkin SD & Martuza RL 2009 Oncolytic herpes simplex virus vectors and taxanes synergize to promote killing of prostate cancer cells. *Cancer Gene Therapy* **16** 551–560. (doi:10.1038/cgt.2009.10)
- Prigent C & Dimitrov S 2003 Phosphorylation of serine 10 in histone H3, what for? *Journal of Cell Science* **116** 3677–3685. (doi:10.1242/jcs.00735)
- Qi G, Ogawa I, Kudo Y, Miyauchi M, Siriwardena BS, Shimamoto F, Tatsuka M & Takata T 2007 Aurora-B expression and its correlation with cell proliferation and metastasis in oral cancer. *Virchows Archiv* **450** 297–302. (doi:10.1007/s00428-006-0360-9)
- Schweppe RE, Klopper JP, Korch C, Pugazhenth U, Benezra M, Knauf JA, Fagin JA, Marlow LA, Copland JA, Smallridge RC et al. 2008 Deoxyribonucleic acid profiling analysis of 40 human thyroid cancer cell lines reveals cross-contamination resulting in cell line redundancy and misidentification. *Journal of Clinical Endocrinology and Metabolism* **93** 4331–4341. (doi:10.1210/jc.2008-1102)
- Seidman MA, Hogan SM, Wendland RL, Worgall S, Crystal RG & Leopold PL 2001 Variation in adenovirus receptor expression and adenovirus vector-mediated transgene expression at defined stages of the cell cycle. *Molecular Therapy* **4** 13–21. (doi:10.1006/mthe.2001.0414)
- Smallridge RC, Marlow LA & Copland JA 2009 Anaplastic thyroid cancer: molecular pathogenesis and emerging therapies. *Endocrine-Related Cancer* **16** 17–44. (doi:10.1677/ERC-08-0154)
- Smith SL, Bowers NL, Betticher DC, Gautschi O, Ratschiller D, Hoban PR, Booton R, Santibáñez-Koref MF & Heighway J 2005 Overexpression of aurora B kinase (AURKB) in primary non-small cell lung carcinoma is frequent, generally driven from one allele, and correlates with the level of genetic instability. *British Journal of Cancer* **93** 719–729. (doi:10.1038/sj.bjc.6602779)
- Sorrentino R, Libertini S, Pallante PL, Troncone G, Palombini L, Bavetsias V, Spalletti-Cernia D, Laccetti P, Linardopoulos S, Chieffi P et al. 2005 Aurora B overexpression associates with the thyroid carcinoma undifferentiated phenotype and is required for thyroid carcinoma cell proliferation. *Journal of Clinical Endocrinology and Metabolism* **90** 928–935. (doi:10.1210/jc.2004-1518)
- Tanaka S, Aarii S, Yasen M, Mogushi K, Su NT, Zhao C, Imoto I, Eishi Y, Inazawa J, Miki Y et al. 2008 Aurora kinase B is a predictive factor for the aggressive recurrence of hepatocellular carcinoma after curative hepatectomy. *British Journal of Surgery* **95** 611–619. (doi:10.1002/bjs.6011)
- Vähä-Koskela MJ, Heikkilä JE & Hinkkanen AE 2007 Oncolytic viruses in cancer therapy. *Cancer Letters* **254** 178–216. (doi:10.1016/j.canlet.2007.02.002)
- Vischioni B, Oudejans JJ, Vos W, Rodriguez JA & Giaccone G 2006 Frequent overexpression of aurora B kinase, a novel drug target, in non-small cell lung carcinoma patients. *Molecular Cancer Therapeutics* **5** 2905–2913. (doi:10.1158/1535-7163.MCT-06-0301)
- Walsby E, Walsh V, Pepper C, Burnett A & Mills K 2008 Effects of the aurora kinase inhibitors AZD1152-HQPA and ZM447439 on growth arrest and polyploidy in acute myeloid leukemia cell lines and primary blasts. *Haematologica* **93** 662–669. (doi:10.3324/haematol.12148)

- Wilkinson RW, Odedra R, Heaton SP, Wedge SR, Keen NJ, Crafter C, Foster JR, Brady MC, Bigley A, Brown E *et al.* 2007 AZD1152, a selective inhibitor of Aurora B kinase, inhibits human tumor xenograft growth by inducing apoptosis. *Clinical Cancer Research* **13** 3682–3688. (doi:10.1158/1078-0432.CCR-06-2979)
- Yang J, Ikezoe T, Nishioka C, Tasaka T, Taniguchi A, Kuwayama Y, Komatsu N, Bandobashi K, Togitani K, Koeffler HP *et al.* 2007 AZD1152, a novel and selective aurora B kinase inhibitor, induces growth arrest, apoptosis, and sensitization for tubulin depolymerizing agent or topoisomerase II inhibitor in human acute leukemia cells *in vitro* and *in vivo*. *Blood* **110** 2034–2040. (doi:10.1182/blood-2007-02-073700)
- Yu W & Fang H 2007 Clinical trials with oncolytic adenovirus in China. *Current Cancer Drug Targets* **7** 141–148. (doi:10.2174/156800907780058817)