AZD1152 negatively affects the growth of anaplastic thyroid carcinoma cells and enhances the effects of oncolytic virus \textit{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 \textit{dl922-947} is active against ATC cells, and we have evaluated the effects of the association between AZD1152 and \textit{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 \textit{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 \textit{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 radiiodine 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).

\textit{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.


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 (1×10⁷) 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 subG$_1$ fraction and G$_2$/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 ≥4N DNA content and a subsequent subG$_1$ 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.
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
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
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 Ser10 (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 1 Simultaneous treatment with AZD1152 and dl922-947 |
|----------------|----------------|----------------|
| **Cell line** | **Ratio** | **EC_{50}** | **EC_{50}** |
|               | ((pfu/cell)/(nM)) | **dl922-947** | **AZD1152** | **Cl** |
| BHT101-5     | AZD1152 alone  | 0             | 13           |
|              | dl922-947 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           |
|              | dl922-947 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  |

| Table 2 AZD1152 pre-treatment on dl922-947-infected cells |
|----------------|----------------|----------------|
| **Cell line** | **Ratio** | **EC_{50}** | **EC_{50}** | **Cl** |
|               | ((pfu/cell)/(nM)) | **dl922-947** | **AZD1152** | **Cl** |
| BHT101-5     | AZD1152 alone  | 0             | 24           |
|              | dl922-947 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          |
|              | dl922-947 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  |
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 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),
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 x 10^6 pfu, in a volume of 300 μl) 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 x 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 x 10^5 pfu, in a volume of 300 μl) 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 x 10^7 pfu, in a volume of 300 μl) 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.
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 phosphorylation of Aurora B on Ser10 (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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