Ionizing radiation enhances dI922–947-mediated cell death of anaplastic thyroid carcinoma cells

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Abstract

dI922–947 is an oncolytic adenovirus potentially suitable for the treatment of aggressive localized tumors, such as anaplastic thyroid carcinoma (ATC). In this study, we have analyzed the effects of dI922–947 in combination with ionizing radiations, testing different schedules of administration and observing synergistic effects only when ATC cells were irradiated 24 h prior to viral infection. Cells undergoing combined treatment exhibited a marked increase in cell death and viral replication, suggesting that irradiation blocks cells in a more permissive state for viral life cycle. We also show that dI922–947 triggers a DNA damage response, characterized by mobilization of the MRN complex (composed by Mre11-Rad50-Nbs1), accumulation of γ H2AX, and activation of the checkpoint kinases ataxia telangiectasia mutated (ATM) and Chk1. Based on these observations, we speculate that the DNA damage response acts as a cellular protective mechanism to hinder viral infection and replication. To confirm this hypothesis, we demonstrate that the ATM inhibitor KU55933 increased the oncolytic activity of dI922–947 and its replication. Finally, we validate the potential therapeutic use of this approach by showing *in vivo* that the combined treatment slows tumor xenograft growth more potently than either irradiation or infection alone.

Key Words

- anaplastic thyroid carcinoma
- oncolytic virus
- irradiation
- DNA damage

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Introduction

Anaplastic thyroid carcinoma (ATC) represents one of the most aggressive human malignancies. It arises from thyroid follicular cells showing morphological features of a malignant neoplasm, high proliferation rate, and marked aneuploidy. The median survival of patients with ATC is ~6 months from diagnosis. Multimodality

therapy, which includes surgical debulking, external radiation therapy, and chemotherapy, has failed to show any improvements in survival and ATC still remains a formidable medical challenge (Smallridge *et al.* 2009). Therefore, novel therapies with different mechanisms of action are required.

Oncolytic viruses (OVs) are replication competent viral mutants able to complete their life cycle exclusively in tumor cells. With respect to classical anti-cancer drugs, OVs present several advantages: tumor-selective replication with amplification of the input dose, stimulation of anti-tumoral immune responses, and relatively few mild side effects when administered to human patients. Also, the development of cross-resistance is unlikely because their mechanism of action is independent of conventional anti-cancer therapeutics (Eager & Nemunaitis 2011, Hallden & Portella 2012). A number of OVs, such as the vaccinia virus GLV-1h68 (Lin et al. 2008b) and the oncolytic attenuated herpes virus G207 (Lin et al. 2008a), have shown promising effects against ATC cells in preclinical studies.

We have previously demonstrated that the oncolytic adenoviruses dl1520 and dl922–947 could be useful for the therapy of ATC (Portella et al. 2002, 2003, Libertini et al. 2007, 2008, 2011). dl1520, bearing a E1B 55K deletion, is the first mutant engineered for tumor-specific cytolysis (Heise et al. 1997). As its replication is severely attenuated not only in normal cells but also in the majority of cancers (O'Shea et al. 2004), dl1520 has shown limited utility as single agent. Hence, novel and more effective OVs have been engineered. dl922–947 is a second-generation adenoviral mutant bearing a 24 bp deletion in E1A-conserved region 2 (CR2; Heise et al. 2000a). This region binds to and inactivates the pRb tumor suppressor protein, dissociating the pRb-E2F complex and driving S-phase entry and viral replication. Because the mutant E1A protein encoded by dl922–947 cannot bind pRb, the virus is unable to trigger S-phase entry in normal cells and can therefore only replicate in cells with an aberrant G1-S checkpoint, a defect observed in over 90% human cancers (Sherr & McCormick 2002). Superior cell killing potency of dl922–947 with respect to dl1520 has been observed *in vitro* and *in vivo* in human cancer cells of different origin such as pancreas (Bhattacharyya et al. 2011), prostate (Radhakrishnan et al. 2010), ovarian (Lockley et al. 2006), thyroid (Libertini et al. 2008), and brain (Yong et al. 2009, Botta et al. 2010, 2012). In addition, other mutants with E1ACR2 region deletion have proved effective in experimental models (Jiang et al. 2009, Oberg et al. 2010).

Emerging evidences indicate that combining OVs with standard chemotherapy and other types of anti-cancer treatments has the potential to increase the antitumor activity (Heise et al. 2000b, Cheong et al. 2008). Preclinical and clinical studies (phase I and II/III) have clearly shown that OVs and radiation therapy show additional or synergistic antitumor effects (Immonen et al. 2004,

Toucheffeu et al. 2011). Despite these encouraging results, relatively little is known about the mechanisms that result in enhanced tumor cell death. Understanding these mechanisms could enable further optimization of this strategy for future clinical development.

Ionizing radiation (IR) is known to induce DNA damage and a subsequent block of the cell cycle progression with accumulation of cells either in G1 or G2, depending on the integrity of cell cycle checkpoints. After DNA repair, these cell cycle checkpoint arrests are released. It has been reported that drugs that block cells in G2/M or inhibit cytokinesis can enhance OV-mediated cell killing (Seidman et al. 2001). For example, we recently demonstrated that the cytopathic effects of dl922–947 are enhanced by the Aurora B inhibitor AZD1152, which induces mitotic arrest, accumulation in G2/M phase, and polyploidy (Libertini et al. 2011).

Here, we have evaluated the effect of dl922–947 in association with IR, showing that the combination enhances the effect of dl922–947 against ATC cells *in vitro*. Comparing different schedules of treatment (virus administered before or after irradiation), we observed synergistic cell killing only when irradiation is administered prior to viral infection. The efficacy of the optimal combined treatment has also been confirmed in ATC tumor xenografts *in vivo*.

Materials and methods

Cells, adenoviruses, and drugs

Human thyroid anaplastic carcinoma cell lines BHT101-5, FRO, and Cal62 have been described and authenticated as shown elsewhere (Schweppe et al. 2008). For growth curve experiments, FRO and BHT101-5 cells were seeded in 12-well culture plates at a density of 10^4 cells/well and, 24 h later, treated as indicated. Cell count was performed using an automatic cell counter (TC-10 Bio-Rad). dl922–947 and AdGFP viral stocks were expanded in the human embryonic kidney cell line HEK-293, purified, stored, and quantified as previously reported (Botta et al. 2010).

zVAD-fmk and KU55933 (both from Tocris Bioscience, Bristol, UK) were dissolved in DMSO to a final concentration of 10 and 5 mM respectively and stored at -20°C . zVAD-fmk was added 2 h prior irradiation.

Cytotoxicity assay

Cytotoxicity was evaluated using the sulforhodamine B assay as described previously (Vichai & Kirtikara 2006).

Dose–response curves were generated to calculate the concentration of each agent required to kill 50% of cells (median lethal dose, LD₅₀). Untreated cells or cells treated with single agents were used as a control.

Viral replication

FRO and BHT101-5 cells were infected with dl922–947, media collected at 48 hours post infection (hpi), and viral DNA was extracted using the High pure viral nucleic acid kit (Roche Diagnostics). Specific primers for the viral hexon gene (from 99 to 242 bp) were used to measure viral replication by real-time PCR: 5'-GCC ACC GAG ACG TAC TTC AGC CTG-3' (upstream primer) and 5'-TTG TAC GAG TAC GCG GTA TCC TCC GCG GTC-3' (downstream primer). A standard curve was constructed by assaying serial dilutions of dl922–947.

For tissue culture infectious dose 50% (TCID₅₀) assays, 10⁵ cells were irradiated with 5 Gy and, after 24 h, infected with five multiplicity of infection (MOIs) (FRO cells) or one MOI (BHT101-5) of dl922–947. At 24 hpi, cells were harvested and subjected to three rounds of freezing/thawing and then centrifuged. The supernatant was titrated on HEK-293 cells by serial dilution (Connell *et al.* 2011). Viral replication in tumor xenografts was evaluated as described previously (Libertini *et al.* 2008).

FACS analysis

Cell cycle FRO and BHT101-5 cells treated as indicated were collected and fixed in 70% (v/v) ethanol for at least 1 h at –20 °C. Washed pellets were resuspended in PBS containing RNaseA (Roche) (0.4 U) and propidium iodide (PI; Sigma) (0.015 mol/l), incubated for 20 min at room temperature, and analyzed for emission in the FL3 channel. To remove artifacts – doublets and aggregates – from the analysis, an electronic doublet discrimination was performed using the area and width of the fluorescence (FL3) pulse.

AdGFP infection FRO and BHT101-5 cells were infected with AdGFP (25 MOIs) and harvested 48 h later. Pellet was resuspended in PBS and emission in the FL1 channel was analyzed.

Annexin V/PI staining Attached and detached FRO and BHT101-5 cells were collected and washed with Annexin V binding buffer (Biolegend, San Diego, CA, USA). Pellet was resuspended in 50 µl of Annexin V

binding buffer containing 2 µl FITC-conjugated Annexin V (Biolegend, #640906). After 15 min of incubation at room temperature, 250 µl PI solution 0.0015 mol/l in PBS was added to each sample just before analysis.

γH2AX/PI and pH3/PI stainings Cells were fixed in 70% (v/v) ethanol for 2 h at –20 °C. Pellet was washed with PBS/Tween buffer (PBT) (0.5% w/v BSA and 0.1% v/v Tween 20 in PBS) and resuspended in PBT containing γH2AX antibody (Millipore, #05-636, 1:100, Billerica, MA, USA) or p-histone H3 (ser10) antibody (Millipore, #06-570, 1:100). After 1-h incubation at room temperature, samples were washed in PBT and then resuspended in PBT containing Alexa488 anti-mouse (Invitrogen, #A11001, 1:100) or Alexa488 anti-rabbit (Invitrogen, #A11008, 1:100) at darkness. After further 30 min, cells were washed with PBT and resuspended in PI 0.015 mol/l for 20 min and analyzed for the emission in FL1 and FL3 channels. All samples were acquired with a BD LSRFortessa (BD Biosciences, San Jose, CA, USA) and analyzed using BD FACSDiva Software.

Protein extraction and western blot analysis

After the indicated treatments, attached and detached cells were harvested and lysates were prepared as already described (Botta *et al.* 2010). Fifty micrograms of protein lysates were probed with the following antibodies: caspase 3 (Abcam, #Ab13585, 1:500, Cambridge, UK), p-ATM s1981 (Cell Signaling, #4526, 1:1000, Boston, MA, USA), p-chk1 s345 (Cell Signaling, #2348, 1:1000), MRE11 (SantaCruz, #sc-5859, 1:1000, Santa Cruz, CA, USA), cyclin B1 (SantaCruz, #sc-245, 1:1000), and α-tubulin (Sigma, #T9026, 1:5000).

Tumorigenicity assay

FRO cells (1 × 10⁶) were injected into the right flank of 80 athymic mice. After 30 days, tumor volume was evaluated and the animals were divided into four groups (20 animals/group) with similar average tumor size. At day 0, a single radiation dose of 10 Gy was administered on the tumor volume at a distance of 80 cm with a bolus interposition to avoid lower doses at the tumor external edge. dl922–947 (2 × 10⁶ MOIs) was injected in the peritumoral area administered three times per week by intratumoral injection to avoid first pass effect in two groups. The control group was injected with saline solution. Tumor diameters were measured with caliber and tumor volumes (V) were calculated by the formula of

rotational ellipsoid: $V = A \times B^2 / 2$ (A , axial diameter; B , rotational diameter).

Mice were maintained at the Dipartimento di Biologia e Patologia Cellulare e Molecolare Animal Facility. All 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. Study was approved by our institutional committee on animal care.

Statistical analysis

The analysis of the cytotoxic effect *in vitro* was made by isobolograms generated to calculate the combination index (CI), as described previously (Tallarida 2001). The isobologram method is a graphical representation of a two-drug pharmacological interaction and is obtained by selecting a desired fractional cell kill (LD_{50}) and plotting the individual drug doses required to generate that LD_{50} on their respective x - and y -axes. A straight line is then drawn to connect the points. The observed dose combination of the two agents that achieved that particular LD_{50} is then plotted on the isobologram. A CI of 1 indicates an additive effect between two agents, whereas a $CI < 1$ or $CI > 1$ indicates synergism or antagonism respectively. Drug synergy was determined by the isobologram and CI methods as described previously (Tallarida 2001).

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, San Diego, CA, USA). Assessment of differences among the rate of tumor growth in mice was made for each time point of the observation period.

Results

Effects of IR on dl922–947

To determine whether IR could enhance the oncolytic activity of the mutant adenovirus dl922–947, ATC cell lines FRO, BHT101-5, and Cal62 were infected and irradiated. To identify most appropriate treatment sequence, cells were infected and then irradiated after 24 h or *vice versa*. Cell survival was evaluated 7 days after infection.

When cells were infected 24 h after irradiation, CI showed a potent, statistically significant synergy of cell killing in Cal62 and BHT101-5 cells at all combinations. In FRO cells, using the same treatment schedule, a slight synergic effect was observed in two combinations

($CI = 0.95$ for 2 Gy plus 7.5 MOIs and $CI = 0.9$ for 4 Gy plus 5 MOIs) whereas at 8 Gy plus 2 MOIs an additive effect ($CI = 1$) was observed (Fig. 1, upper panels).

In contrast, an antagonist effect was observed in FRO and Cal62 cells when infection was followed by irradiation, whereas in BHT101-5 cells only 4 Gy combined with 2.5 MOIs showed synergy with this schedule (Fig. 1, lower panels). These data demonstrate that the cytotoxicity of dl922–947 is significantly enhanced when cells are irradiated 24 h prior to infection.

Viral entry and viral replication analysis

It has been proposed that radiations could enhance oncolytic activity by increasing viral entry in target cells (Anders *et al.* 2003). To monitor this step, FRO and BHT101-5 cells were irradiated and after 24 h infected with a non-replicating reporter adenovirus transducing GFP (AdGFP). After an additional 48 h, GFP emission was evaluated by cytofluorimetric analysis. As shown in Fig. 2A, irradiation neither increases the percentage of GFP-positive cells nor the average green emission of individual cells, except for the 2 Gy sample, where a slight, albeit significant positive shift in the GFP fluorescence was observed in both cell lines.

Next, a real-time PCR assay was performed to evaluate viral replication. FRO and BHT101-5 cells were irradiated (2–4–5 Gy) and after 24 h infected with different MOIs (1–5–10) of dl922–947. After an additional 48 h, viral genome copies were evaluated by real-time PCR. This analysis revealed that IR induced a significant ($*P < 0.05$) or highly significant ($**P < 0.005$) dose-dependent increase in viral replication (Fig. 2B and C, left panels). A TCID₅₀ assay confirmed an increased viral production in irradiated cells (Fig. 2B and C, right panels).

Analysis of cell cycle profile and cell death

It has been reported that drugs able to block cells in G2/M or inhibit cytokinesis could enhance the effects of OV (Seidman *et al.* 2001). Irradiation is known to induce a transitory block in G2 phase (Lisby *et al.* 2004); therefore, we analyzed cell cycle profiles to evaluate differences in cell cycle phases and timing of cell death. FRO and BHT101-5 cells were irradiated (8 Gy) and, 24 h later, infected with five or one MOIs of dl922–947 respectively. Starting from 6 hpi, cells were collected, stained with PI, and cell cycle analyzed by FACS.

At the time of the infection, that is 24 h after irradiation (24hpiR), most of the cells were in G2/M

Figure 1

IR enhances the cytopathic effects of dl922-947. Combination index of FRO, BHT101-5, and Cal162 cells irradiated and after 24 h infected (upper panels)

or vice versa (lower panels). A synergistic effect was observed only in cells infected 24 h after irradiation (CI < 1).

phase (Fig. 3A), a checkpoint arrest that facilitates DNA repair. Conversely, dl922-947-infected cells accumulated in G2/M phase at 6 hpi but, in contrast to the effect of irradiation alone, at 48 hpi most of the cells were either still in G2/M phase or had become polyploid (Fig. 3A). In cells infected 24 h after irradiation, we observed up to 24 h post-infection G2/M accumulation. However, at 48 hpi, most of the cells escaped from the block and become polyploid (Fig. 3A), showing a cell cycle profile similar to infected cells.

To discriminate between G2 phase and mitotic block, we used two specific biochemical markers: histone H3 phosphorylated on serine 10 (pH3) for mitosis and cyclin B1 for G2 phase (Lao-Sirieix et al. 2004). As shown by FACS analysis in Fig. 3B, the single treatments reduced the levels of the mitotic marker pH3 both at 6 and 24 hpi; this reduction was further increased in the combined treatment. Western blot analysis of cyclin B1 confirmed that single and combined treatments induced in both cell lines a G2 accumulation (Fig. 3C).

Cell cycle analysis showed that cells irradiated prior to infection exhibited an increased percentage of events in the subG1 fraction (Fig. 3A), which is indicative of either necrosis or apoptosis. To discriminate between the two types of cell death, unfixed cells were stained with PI and Annexin V. PI single positivity represents necrosis, single Annexin V staining early apoptosis and double positivity indicates late apoptosis. Infected cells showed an increase in Annexin V positivity and this positivity was further increased by irradiation (Fig. 4A, green numbers). At 72 hpi (Fig. 4A, left), we observed that only 12% of the irradiated/infected cells were alive compared with the 24% of the infected cells and the 54% of the irradiated cells, further confirming the efficacy of the combined treatment. Similar trend was observed in BHT101-5 cells (Supplementary Figure 1A, see section on supplementary data given at the end of this article).

As the increase in Annexin V positivity suggested the induction of an apoptotic pathway, we analyzed caspase 3 activation in FRO and BHT101-5 cells. In both

Figure 2

IR does not affect viral entry but enhances viral replication of *dl922-947*. (A) FACS analysis of AdGFP-infected cells. FRO and BHT101-5 were irradiated (2–4–8 Gy) and after 24 h infected with AdGFP (25 MOIs). The histogram on the left side shows the percentage of GFP-positive cells whereas the mean GFP emission is reported on the right side. Both parameters, with the exception of 2 Gy, were not increased in irradiated cells. The data are the mean of three different experiments (* $P < 0.01$). (B and C) Replication of *dl922-947* was measured by real-time PCR genome

equivalent analysis (left panels) and by TCID₅₀ assay (right panels). For real-time PCR analysis, FRO and BHT101-5 cells were irradiated (2–4–5 Gy) and 24 h later infected with *dl922-947* (1–5–10 MOIs). For TCID₅₀ assay, FRO and BHT101-5 were irradiated (5 Gy) and after 24 h infected with five and one MOIs respectively. Irradiated cells showed significant or highly significant differences in viral replication levels with respect to non-irradiated cells. The data are the mean of three different experiments (* $P < 0.05$; ** $P < 0.005$).

cell lines, a decrease in procaspase 3 and an increase in cleaved caspase 3 were observed in the combined treatment with respect to single treatments (Fig. 4B and Supplementary Figure 1B). To better understand the role of caspases in the cell death mechanisms elicited by the combined treatments, we performed a growth curve in the presence of the pan-caspase inhibitor zVAD-fmk. Addition of the drug 2 h prior IR/*dl922-947* did not modify cell proliferation, suggesting that other cell death pathways are activated by the combined treatment (Fig. 4C and Supplementary Figure 1C).

DNA damage repair system and *dl922-947* infection

IR induces DNA damage and, after its detection, cellular pathways are activated to halt cell cycle progression and repair the damage. Adenoviral replication also induces DNA damage (Toucheffeu *et al.* 2011); therefore, we analyzed in FRO cells the effects of viral infection, alone or in combination with radiation, on DNA damage repair system.

The MRN complex (composed by Mre11-Rad50-Nbs1) acts as a double-strand breaks (DSBs) sensor. It localizes to

Figure 3

IR induces a G2/M accumulation that is further increased by *dl922-947* infection. (A) Cell cycle analysis of FRO and BHT101-5 cells. Sub-G1, G2/M, and polyploidy are represented by histograms. hpiR, hours post irradiation; hpi, hours post infection. The data are the mean of three different experiments. (B) Mitotic index. FRO and BHT101-5 cells were irradiated with 8 Gy and after 24 h infected with five or one MOIs of *dl922-947*

respectively. pH3 levels were quantified at 72 hpi. Both IR and virus alone induced a drop in pH3 levels, which is more dramatic in the combined treatment. The data are the mean of three different experiments. (C) Western blot analysis of cyclin B1 levels. The data are the mean of three different experiments.

DNA breaks and activates the ATM-dependent signaling pathway, which coordinates cell cycle arrest and DNA repair (Lavin 2007). Western blot analysis showed a strong reduction of Mre11 levels in infected cells, most probably due to protein degradation. The level of Mre11 was not altered in irradiated cells; however, a similar decrease occurred when cells were subject to both irradiation and infection (Fig. 5A). Cells treated with virus, alone or in combination with IR, also showed a strong activation of ATM at 24 hpi, whereas irradiated cells showed an earlier activation (1–3 hpi), reversed to basal levels by 24 hpi (Fig. 5A).

Activation of the checkpoint kinase Chk1, as judged by phosphorylation on serine 345 (S345) (Smith et al. 2010), showed strong induction at early points (1–6 h) that also declined to control levels by 24 after irradiation. In marked contrast, infection with *dl922-947* induced robust Chk1 phosphorylation at 24 hpi, regardless of prior irradiation (Fig. 5A).

To quantify DNA damage, we evaluated γ H2AX (histone H2AX phosphorylated at serine 139) levels by cytofluorimetric analysis of both the percentage of positive cells (shown in green in Fig. 5B) and the

median fluorescence value of the positive population (in red). After irradiation, γ H2AX levels peaked after 3 h and then progressively declined, confirming that irradiation-induced DNA damage was repaired within 24 h. In infected cells, γ H2AX levels raised after 3 hpi and, by 24 hpi, most of the cells showed strong γ H2AX positivity, indicating progressive accumulation of DNA damage (Fig. 5B). In cells subject to irradiation and infection together, an earlier increase in γ H2AX levels was observed. However, at 24 hpi, γ H2AX levels remained as high as in cells infected with *dl922-947* alone.

H2AX is phosphorylated at Ser139 by various kinases, including ATM, ATR, and DNA-PK, all of which were reported to be involved in the responses of mammalian cells to DSB. At odds with γ H2AX levels, infected cells and cells undergoing the combined treatment showed a strong induction of ATM only at 24 hpi, indicating an ATM-independent phosphorylation of H2AX. It has been reported an ATR activation in response to replicative stress (Ward & Chen 2001). To understand whether the increase in γ H2AX levels could be caused to the activation of ATR in response to *dl922-947*-induced replicative stress, we evaluated H2AX phosphorylation in the different phases

Figure 4

IR increases the cell death induced by *dl922-947*. (A) Annexin V/PI staining. FRO cells were irradiated with 8 Gy and after 24 h infected with five MOIs of *dl922-947* for 72 h. Apoptotic cells (right quadrant) are positive for Annexin V (early apoptosis) or double positive for Annexin V and PI (late apoptosis); necrotic cells are only positive for PI (upper left panel); vital cells are double negative (lower left panel). Tables show the percentage of the different stained population after 24 and 48 h of treatment. The data are the mean of

three different experiments. (B) Western blot analysis of procaspase 3 and caspase 3 levels. Arrows indicate the bands corresponding to the different forms of cleaved caspase 3. The data are the mean of three different experiments. (C) Growth curve analysis performed in FRO cells treated 2 h prior the combined treatment irradiation/*dl922-947* infection with the pan-caspase inhibitor *zVAD-fmk*. The treatment with *zVAD-fmk* showed no effects on cell survival. These data are the mean of three different experiments.

of cell cycle. γ H2AX was mainly observed in replicative and post-replicative phases (S-phase and in the following G2/M and polyploid cells with $>4N$ DNA content) (Fig. 5B), supporting a correlation between γ H2AX levels and viral induced replicative stress as reported previously (Connell et al. 2011).

Pharmacological inhibition of ATM activation and *dl922-947* infection

The reduction of Mre11 levels together with ATM phosphorylation suggests that viral genomes are recognized as DNA damage in infected cells and that viral function may target the MRN complex to prevent DNA repair processes interfering with viral replication.

To test whether activation of ATM signaling pathway hampers viral replication, infected cells were treated with a pharmacological inhibitor of ATM, KU55933, whose specificity has been demonstrated by the ablation DNA

damage-induced phosphorylation of a range of ATM substrates, including p53 and H2AX (Hickson et al. 2004).

FRO and BHT101-5 cell lines were infected in combination with two different concentrations of the drug (5–10 μ M). As shown in Fig. 6A, KU55933 treatment potentiates the cytotoxic effects of *dl922-947*. As KU55933 treatment alone did not show any cytotoxic effect on both cell lines, CI was not evaluated.

We also examined the effect of KU55933 on γ H2AX formation in infected FRO and BHT101-5 cells. As shown in Fig. 6B and in Supplementary Figure 2, see section on supplementary data given at the end of this article, KU55933 only partially inhibited viral induced H2AX phosphorylation, further supporting ATM-independent mechanism(s) for γ H2AX formation as discussed in the previous paragraph. Genome equivalent copies of *dl922-947* in cells treated with KU55933 showed that the drug significantly enhanced viral replication in a dose-dependent manner (Fig. 6C).

A

B

FRO cells

Figure 5

dl922-947 infection activates the DNA damage signaling pathway and impairs DNA damage repair. (A) Western blot analysis of p-ATM (serine 1981), pChk1 (serine 345), and Mre11 levels. (B) FACS analysis of γH2AX and cell cycle. FRO cells were irradiated (8 Gy) and infected with dl922-947

(five MOIs) for 1–3–6–24 h. Histograms on the right side represent the percentage of γH2AX-positive and γH2AX-negative cells in the different phase of the cell cycle.

IR increases dl922-947 oncolytic activity *in vivo*

To evaluate the therapeutic potential of synergism between dl922-947 and IR, we analyzed the effects of the combined treatment on xenograft tumors.

Athymic mice were inoculated subcutaneously with FRO cells. Two groups were irradiated with a single radiation dose (10 Gy) on the tumor volume. A low viral dose (2×10^6 pfu) was used to better visualize the effects of the combined treatment vs dl922-947 infection alone. As

Figure 6

KU55933 enhances the cytopathic effects of *dl922–947*. (A) FRO and BHT101-5 cells were treated with KU55933 (5–10 μM) and infected at increasing MOIs (1–2.5–5–10 for FRO cells and 0.5–1–2.5–5 for BHT101-5 cells). KU55933 was added every 24 h to exclude the effect of a rapid drug catabolism. Survival was evaluated after 7 days by sulforhodamine B method. Data are the mean of three different experiments. (B) FRO cells were treated with KU55933 (5 μM) and infected with *dl922–947* (five MOIs) for 3–6–12–24 h. Fixed cells stained with γH2AX and propidium iodide were analyzed using FACS. Percentage of γH2AX -positive cells (green) and

median of FITC emission (red) are shown. Treatment with the ATM inhibitor reduces *dl922–947*-induced γH2AX levels, particularly as median of FITC emission. The data are the mean of three different experiments. (C) Real-time PCR genome equivalent analysis. ATC cells were treated with KU55933 (0.5–1–5 μM) and then infected with *dl922–947* (5–10 MOIs). A highly significant difference in viral replication levels was observed with respect to cells not treated with KU55933 (* $P < 0.005$; ** $P < 0.0001$). The data are the mean of three different experiments.

Figure 7

The combined treatment IR plus *dl922-947* reduces tumor growth. (A) The difference between animals receiving the combined treatment and single treatment groups and control group becomes statistically significant ($*P < 0.05$) from day 11. At day 21, a highly significant difference ($**P < 0.005$) was observed. (B) Real-time PCR genome equivalent analysis

in tumor xenografts. A highly significant difference ($**P < 0.005$) in viral replication levels was observed in irradiated tumors. (C) Western blot analysis of procaspase 3 and γ H2AX levels. Numbers represent the result of the densitometric analysis.

shown in Fig. 7A, the combined treatment group showed a significant reduction of tumor growth with respect to single treatments and to the untreated group. This difference was first evident by day 11, and by day 21, growth inhibition was highly statistically significant compared with control group (Fig. 7A). Genome equivalent copies were evaluated by real-time PCR: in tumors receiving the combined treatment, a higher viral DNA content was observed (Fig. 7B). Western blot analysis showed a strong γ H2AX accumulation and an increased caspase 3 activation in the combined treatment group compared with single treatment groups (Fig. 7C).

Discussion

ATC represents one of the most aggressive human malignancies with a poor prognosis, and its treatment

still represents a major challenge (Smallridge *et al.* 2009). Currently available treatments are considered palliative because of poor response rates and lack of demonstrable survival benefits, highlighting the need for more effective treatments (Smallridge *et al.* 2009, Taccaliti *et al.* 2012).

OVs represent a promising approach as anti-cancer therapeutics; however preclinical and clinical studies have demonstrated limited efficacy of OVs alone, prompting effort to combine them with other anti-cancer agents to improve their antitumor effects (Eager & Nemunaitis 2011, Hallden & Portella 2012). We have previously demonstrated that the oncolytic adenoviruses *dl1520* and *dl922-947* are active against ATC *in vitro* and *in vivo* (Libertini *et al.* 2007, 2008).

Radiotherapy is frequently used for the treatment of ATC (Taccaliti *et al.* 2012), and we have also demonstrated that mutated adenovirus *dl1520*, despite its limited

efficacy, enhances the effects of IR against ATC cells and xenograft tumors (Portella *et al.* 2003). Here, we have analyzed the effects of a more potent second-generation OV, dl922–947, in combination with IR. We show that both the cytopathic activity and replication of dl922–947 are significantly enhanced by IR.

The potentiating effect of irradiation on the cytopathic potential of OVs, other than dl922–947, has already been demonstrated. However, optimal strategies for combining these therapeutic modalities still remain to be established as evidence suggests that they interact in complex ways that vary in a cell line- or virus-dependent fashion (Toucheffeu *et al.* 2011). Here, we have identified the most effective treatment sequence for ATC cells showing that irradiation prior to dl922–947 infection results in enhanced viral replication and synergistic cell killing. Irradiation after viral infection did not show synergistic or even additive effects.

It has been proposed that irradiation can increase viral uptake (Zhang *et al.* 2003) either by enhancing coxsackie and adenovirus receptor (CAR) and integrin expression on cell membranes or by modulating the expression of Dynamin 2, an intracellular protein involved in adenoviral internalization (Qian *et al.* 2005). In a study using a telomerase-specific replication selective adenovirus (OBP-301), viral infection was shown to result in radiosensitivity in A459 small-cell lung cancer cells (Kuroda *et al.* 2010). In the same study, a dose-dependent increase in adenoviral uptake and coxsackie adenovirus receptor expression was also observed.

By infecting the cells with an AdGFP virus, we evaluated the effects of IR on viral uptake on ATC cells. In contrast to previous studies, we observed only very modest effects of IR on viral uptake. At 2 Gy, a small increase was observed, whereas higher radiation doses do not significantly modify viral uptake. Moreover, CAR receptor levels were not modified by IR (data not shown). Differences in treatment sequence, virus, and cell lines may explain the discrepancies with other studies.

Despite this, we did observe a significant increase in viral replication in irradiated cells. Existing data regarding the effect of radiation on viral replication are conflicting. Enhanced viral replication following IR has been reported in some studies (Liu *et al.* 2007, Bieler *et al.* 2008), but not in others (Lamfers *et al.* 2002, Geoerger *et al.* 2003), suggesting that increased viral replication is not an absolute prerequisite for enhanced cell killing.

Subversion of the host cell cycle is an important feature of adenoviral infection. The expression of E1A gene products drives an unscheduled DNA synthesis

followed by an abortive cytokinesis, inducing G2 accumulation and >4N DNA content (Davy & Doorbar 2007). The potential benefit of the G2 accumulation is represented by the pseudo S phase that maintains host cells in a replicative state (Davy & Doorbar 2007, Nichols *et al.* 2009). It has been proposed that drugs blocking cells in G2 phase could enhance the effects of OVs. Indeed, we have demonstrated that the inhibitor of the mitotic kinase Aurora B, AZD1152, enhances the effects of dl922–947 by inducing a G2 arrest (Libertini *et al.* 2011). Accordingly, the accumulation in G2 phase induced by radiation likely enhances the cytopathic effects of dl922–947. This hypothesis is further supported by the observation that infection followed by irradiation did not potentiate cell killing or viral replication (data not shown).

We have analyzed the effects of the combined treatment, showing an increase in number of cells in sub-G1 phase and a sharp reduction in the number of vital cells with respect to single treatments. Infection alone also triggered similar effects, although with lesser efficiency. In the present work, we observed that dl922–947, alone or in combination with IR, induced phosphatidylserine exposure and caspase 3 cleavage, suggesting the activation of apoptosis. However, the treatment with the caspase inhibitor zVAD-fmk was not able to restore cell proliferation, supporting the involvement of other cell death pathways rather than the classical apoptosis. Our results are in agreement with the previous reports suggesting alternative cell death mechanisms induced by dl922–947 (Abou El Hassan *et al.* 2004, Baird *et al.* 2008, Libertini *et al.* 2011).

It is known that the cells respond to adenovirus infection by activating a DNA damage response (Lilley *et al.* 2007, Nichols *et al.* 2009, Toucheffeu *et al.* 2011); indeed, it has been proposed that the prolonged S-phase induced by the virus could resemble the cellular environment occurring in response to replication-associated DNA damage (Connell *et al.* 2011). Also, the linear adenovirus genome has double-stranded DNA termini that could represent targets for cellular DSB repair pathways. IR itself induces DNA damage; therefore, we analyzed the effects of the combined treatment on key components of DSB pathway. In ATC-irradiated cells, DNA damage is correctly repaired, as demonstrated by the progressive reduction toward basal levels of γ H2AX at 24 h after irradiation. Conversely, upon infection with dl922–947, an inefficient DNA damage response was observed. Indeed, despite the expected ATM and Chk1 activation, γ H2AX accumulation was still revealed at 24 hpi, indicating the presence of an unrepaired DNA. The strong reduction of Mre11 levels

observed 24 h after the infection, likely due to degradation induced by viral proteins (Nichols *et al.* 2009), and the viral induced replicative stress could explain the accumulation of an unrepaired DNA. A similar inadequate DNA damage response was reported in cells subject to irradiation prior to infection. However, a more rapid increase in γ H2AX-positive cells was seen, probably due to IR-induced DNA damage combined with that induced by viral replication.

A strong activation of ATM was observed only at 24 hpi, whereas γ H2AX levels rose already at 3 hpi, suggesting a ATM-independent phosphorylation of H2AX as previously reported (Ward & Chen 2001, Nichols *et al.* 2009). H2AX is phosphorylated by various kinases other than ATM, including ATR that activates Chk1. Chk1 is phosphorylated as early as 3 hpi, indicating a temporal correlation between γ H2AX accumulation and ATR–Chk1 activation. This observation is in agreement with previous studies showing a robust induction of ATR–Chk1 pathway upon infection with wild-type and mutant adenoviruses, including dl922–947 (Connell *et al.* 2011). Moreover, our data suggest a correlation with the replicative stress induced by the virus, as γ H2AX is mainly observed in replicative and post-replicative phases.

During the infection with the telomerase-driven oncolytic adenovirus OBP301, the degradation of MRN complex was accompanied by greatly reduced levels of ATM activation (Kuroda *et al.* 2010). It is possible that dl922–947 infection is not able to fully suppress the early events in DNA damage signaling, as MRN complex degradation may not in itself be sufficient to preclude/reduce ATM and ATR kinase activation, as previously reported (Nichols *et al.* 2009). Several reports indicate that ATM activation counterbalances viral infection (Nichols *et al.* 2009); therefore, we postulated that ATM activation could be detrimental for viral infection/replication. The increased cell death observed in cells infected in the presence of the specific ATM inhibitor KU55933 confirmed this hypothesis.

It has been shown that the block of ATR–Chk1 pathways augments dl922–947 cytotoxicity (Connell *et al.* 2011). In this study, we demonstrated that also ATM inhibition could represent an approach to increase dl922–947 cytotoxicity. Our data, together with previous studies, indicate that the DNA damage response pathway acts as an intrinsic cellular defense against the virus and could be targeted to potentiate the effects of oncolytic adenovirus.

Finally, we confirmed the therapeutic potential of combining irradiation with dl922–947 infection using

xenografts formed from ATC cells. Although the s.c. implant of neoplastic cells do not fully reproduce the stromal response and the pattern of spread of the neoplastic cells to lymph nodes and other metastatic sites as in orthotopic models, the data obtained clearly demonstrate the efficacy of the combination therapy *in vivo*.

A majority of patients with ATC die from aggressive local regional disease, primarily from upper airway respiratory obstruction (Taccaliti *et al.* 2012). For this reason, radiotherapy has been used to control local growth, evolving from palliation on to preoperative or/and postoperative therapy to prolong survival.

Given the importance of radiotherapy in the control of ATC, our results suggest that dl922–947 could be combined with this modality in order to improve local control and offer the possibilities of new clinical trials for this intractable disease.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0001>.

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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