An engineered avian-origin influenza A virus for pancreatic ductal adenocarcinoma virotherapy

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Pancreatic ductal adenocarcinoma (PDA) is one of the leading causes of cancer-related deaths worldwide and the development of new treatment strategies for PDA patients is of crucial importance. Virotherapy uses natural or engineered oncolytic viruses (OVs) to selectively kill tumour cells. Due to their genetic heterogeneity, PDA cells are highly variable in their permissiveness to various OVs. The avian influenza A virus (IAV) H7N3 A/turkey/Italy/2962/03 is a potent inducer of apoptosis in PDA cells previously shown to be resistant to other OVs (Kasloff et al., 2014), suggesting that it might be effective against specific subclasses of pancreatic cancer. To improve the selectivity of the avian influenza isolate for PDA cells, here confirmed deficient for IFN response, we engineered a truncation in the NS1 gene that is the major virus-encoded IFN antagonist. The recombinant virus (NS1-77) replicated efficiently in PDA cells, but was attenuated in non-malignant pancreatic ductal cells, in which it induced a potent IFN response that acted upon bystander uninfected cancer cells, triggering their death. The engineered virus displayed an enhanced ability to debulk a PDA-derived tumour in xenograft mouse model. Our results highlight the possibility of selecting an IAV strain from the diverse natural avian reservoir on the basis of its inherent oncolytic potency in specific PDA subclasses and, through engineering, improve its safety, selectivity and debulking activity for cancer treatment.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) is considered as one of the most lethal malignancies in humans. Late detection, early metastases, difficult surgical approach and resistance to chemotherapy all contribute to one of the worst prognoses among the various gastrointestinal cancers with a 5-year survival rate of merely 6% (American Cancer Society, 2013). Development of new treatment strategies for patients suffering from PDA is an urgent necessity.

Pancreatic cell carcinogenesis develops through accumulation of a characteristic set of mutations and genetic lesions which lead to activation of oncogenes and inactivation of tumour suppressor genes (KRAS, CDKN2A, TP53 and SMAD4/DPC4) (Bardeesy & DePinho, 2002). A heterogeneous collection of secondary genetic alterations is also present in patients with pancreatic cancer (Biankin et al., 2012; Yachida & Iacobuzio-Donahue, 2013), giving rise to various PDA subclasses, reflected in a variety of cancer-derived cell lines (Deer et al., 2010), which may display different therapeutic responsiveness and thus may require ad hoc intervention strategies (Biankin et al., 2012; Collisson et al., 2011).

Virotherapy is a treatment based on oncolytic viruses (OVs) that exploit the genetic aberrations in tumours to selectively or preferentially infect, replicate and ultimately kill cancer cells whilst exerting minor or no effect on the surrounding healthy cells (Russell et al., 2012). The translational impact of this technique has been further strengthened by the approval of the adenovirus H101 for the treatment of head and neck cancer patients in China (Garber, 2006), by the registration of the ECHO-7 virus.
RIGVIR for the treatment of melanoma in Latvia and Georgia and more recently by the licensing of the herpes simplex virus Talmigenove laverparepvec (T-Vec) for the treatment of melanoma in the USA (U.S. Food & Drug Administration, 2015). However, so far PDA cells have displayed mixed susceptibility to OVs (Lee et al., 2014; Moerdyk-Schauwecker et al., 2012; Murphy et al., 2012; Wennier et al., 2011), underscoring that virotherapy for tumours which tend to be heterogeneous in nature should not rely on a short list of possible candidates.

Avian-origin influenza A viruses (IAVs) were previously shown to infect and induce potent apoptosis of various PDA cell lines, including BxPC-3 cells (Kasloff et al., 2014) in which different viruses displayed reduced oncolytic effect compared to other malignant pancreatic cells (Lee et al., 2014; Murphy et al., 2012). These observations suggested that avian influenza isolates might be a useful treatment option for specific subclasses of PDA. As such, the low pathogenicity (LP) avian IAV H7N3 A/turkey/Italy/2962/03, whose rapidity and potency of cell death induction in different PDA cell lines repeatedly outperformed other IAV isolates (Kasloff et al., 2014), including the human H1N1 PR8 virus currently tested as oncolytic candidate for other tumour types (Sturlan et al., 2010; van Rikxoort et al., 2012; Wolschek et al., 2011), was selected for further development.

To improve cancer selectivity, OVs can be engineered to exploit the defective antiviral defences of tumour cells (Naik & Russell, 2009). Loss of function of the cyclin-dependent kinase inhibitor 2A gene (CDKN2A), on chromosome 9p21, brought by mutation or homozygous deletion occurs in 80–95% of sporadic pancreatic adenocarcinomas (Hruban et al., 2000; Rozenblum et al., 1997) and is considered an early event in the progression of pancreatic neoplasm. Homozygous deletion of the genetic material that leads to the CDKN2A gene inactivation is also responsible for the partial or complete loss of both copies of IFN-α/β genes located on the same chromosome band and for the ensuing impaired expression of type I IFN often reported for PDA cells (Chen et al., 1996; Ghadimi et al., 1999; Vitale et al., 2007). Thus, mutations that compromise the virus’ ability to counteract IFN-mediated antiviral response should result in preferential virus replication in cancer cells.

The IAV non-structural protein 1 (NS1) is a multifunctional protein, whose major role is to antagonize host IFN-mediated antiviral responses (Hale et al., 2008). The NS1 protein is notionally divided into two distinct functional domains: an N-terminal double-stranded RNA (dsRNA)-binding domain (RBD; aa 1–73) and a C-terminal effector domain (ED; aa 88–C terminus), connected via a short inter-domain linker region (LR) (Bornholdt & Prasad, 2008; Hale, 2014). Both domains contribute in different ways to counteract host IFN responses by sequestering viral RNA from cellular sensors (Donelan et al., 2003), inhibiting the activation of RIG-I by binding to TRIM25 (Gack et al., 2009), inhibiting post-transcriptional processing of host-cell mRNAs (Noah et al., 2003) and blocking the function of cytoplasmic antiviral proteins 2′–5′-oligoadenylate synthetase (OAS) (Min & Krug, 2006) and dsRNA-dependent serine/threonine protein kinase R (PKR) (Li et al., 2006).

IAVs that lack a functional NS1 (ΔNS1) fail to replicate efficiently in normal cells. However, in cancer cells with deficient IFN responses, such mutants have been shown to replicate and cause cell death (Bergmann et al., 2001; Muster et al., 2004). A recombinant influenza virus with NS1 truncation rather than complete deletion was less attenuated and shown to be more effective as an oncolytic treatment in xenograft mouse model of human melanoma (Muster et al., 2004).

Therefore, to obtain a virus that could replicate efficiently in IFN-deficient PDA cells but not in healthy IFN-competent cells, we introduced mutations within the IAV NS1 gene to create a truncated protein of just 77 aa (NS1-77). We tested the virus engineered in this way for oncolytic properties in PDA cell culture and in a mouse xenograft model.

RESULTS AND DISCUSSION

NS1-77 truncation enhances H7N3 driven IFN expression in non-malignant pancreatic ductal cells

Like many cancer cell lines, BxPC-3 cells were shown to be compromised in type I IFN expression due to chromosomal aberrations (Chen et al., 1996; Ghadimi et al., 1999; Hruban et al., 2000; Vitale et al., 2007). To limit viral replication in IFN-competent healthy cells but not in IFN-impaired tumour cells, the H7N3 NS1 gene was mutated to produce a protein of only 77 aa (NS1-77), resulting in the complete loss of the ED (Hale et al., 2008). A second NS1-77 truncated IAV was also generated using the genetic backbone of the well-studied laboratory human strain H1N1 A/Puerto Rico/8/34 (PR8), previously investigated for use as an OV against other types of cancers (Bergmann et al., 2001; Muster et al., 2004; Sturlan et al., 2010).

The ability of the two pairs of IAVs to counteract the host IFN-mediated antiviral response was first assessed in A549-IFNLuc cells, a human lung cell line that expressed a reporter construct in which the luciferase gene was downstream of an IFN-β promoter (Hayman et al., 2006). Results showed that the wild-type H7N3 virus efficiently controlled IFN induction as no difference in luciferase expression was observed compared to the mock infection (Fig. 1a). However, the H7N3 NS1-77 virus induced a significantly higher signal than the mock infection or infection with the H7N3 wild-type virus. Interestingly, while the truncation of the NS1 ED in this avian influenza virus led to a strong reduction of the ability to limit IFN expression, the difference in the IFN
expression induced by PR8 viruses with full length or truncated NS1 proteins was less pronounced.

The IFN responses from BxPC-3 or HPDE6 cells were then tested by infecting each cell type with an equal multiplicity of recombinant IAV bearing full length or truncated NS1 genes. A biological measure of secreted IFN was performed by transferring UV-irradiated supernatants derived from infected cells onto 293T cells previously transfected with ISG54Luc (firefly) and RenillaLuc (Renilla) reporter plasmids, by supernatants obtained from HPDE6 or BxPC-3 cells infected with H7N3 or PR8 recombinant viruses bearing full length or truncated NS1 protein. Results are shown as firefly/Renilla luciferase activity ratio. (c) Luciferase expression driven by pCAGGSLuc plasmid in 293T cells following co-transfection with plasmids encoding full length or truncated NS1 proteins from H7N3 and PR8 viruses. Results are expressed as RLU. (d) Western blot targeting V5 tag in the cell lysates following transfection with the different pCAGGS/NS1 expression plasmids. Vinculin was used as a loading control. All data represent means±SD of one representative experiment (n=3). Statistical significance was determined using one-way ANOVA followed by Bonferroni’s multiple comparison test.

**Fig. 1.** Strain specific effect of NS1-77 truncation on IFN expression in infected cells. (a) IFN-β expression in A549-IFNLuc cells infected with H7N3 and H1N1 PR8 reverse genetics viruses bearing full length or truncated NS1 protein. Results are shown as raw data and expressed as RLU (relative light units). (b) Luciferase expression induced in 293T cells, transfected with ISG54Luc (firefly) and RenillaLuc (Renilla) reporter plasmids, by supernatants obtained from HPDE6 or BxPC-3 cells infected with H7N3 or PR8 recombinant viruses bearing full length or truncated NS1 protein. Results are shown as firefly/Renilla luciferase activity ratio. (c) Luciferase expression driven by pCAGGSLuc plasmid in 293T cells following co-transfection with plasmids encoding full length or truncated NS1 proteins from H7N3 and PR8 viruses. Results are expressed as RLU. (d) Western blot targeting V5 tag in the cell lysates following transfection with the different pCAGGS/NS1 expression plasmids. Vinculin was used as a loading control. All data represent means±SD of one representative experiment (n=3). Statistical significance was determined using one-way ANOVA followed by Bonferroni’s multiple comparison test.

rise to ISG54 promoter stimulation even when infected by a virus with truncated NS1, confirming their defect in IFN response (Fig. 1b). Conversely, supernatants from infected HPDE6 cells triggered luciferase signals from the ISG54 promoter that were significantly higher following infection with the H7N3 NS1-77 deletant virus. In line with the previous data, the recombinant PR8 NS1-77 virus was not affected as much as H7N3 NS1-77 by the truncation, since it did not show any significant difference in ISG54 stimulation compared to its wild-type virus counterpart (Fig. 1b).
The greater impact of NS1 truncation on the ability of the H7N3 virus to control IFN induction implies that this NS1 protein relies on its C-terminal ED to post-transcriptionally limit processing of host pre-mRNAs (Geiss et al., 2002; Hayman et al., 2006; Kochs et al., 2007), whereas the NS1 protein of H1N1 PR8 virus does not due to amino acid substitutions that block its interaction with the cleavage and polyadenylation specificity factor 30 (CPSF30) (Kochs et al., 2007; Steidle et al., 2010). To monitor the expression of a gene transcribed by host-cell machinery, 293T cells were transfected with pCAGGSLuc plasmid, which directs polymerase II mediated expression of firefly luciferase. In addition, to measure the effect of the different NS1 proteins, pCAGGS/PR8 NS1, pCAGGS/H7N3 NS1, pCAGGS/H7N3 NS1-77 or empty pCAGGS (K/C0) plasmids were co-transfected. The expression of luciferase in 293T cells was dependent on the ability of the different NS1 proteins to interfere with the maturation of the luciferase pre-mRNA. Results showed that full length H7N3 NS1 (pCAGGS/H7N3 NS1) inhibited the expression of the reporter luciferase gene from the co-transfected pCAGGSLuc, whereas expression of full length PR8 NS1 protein (pCAGGS/PR8 NS1) did not (Fig. 1c). The truncation of the ED from the H7N3 NS1 (pCAGGS/H7N3 NS1-77) resulted in the loss of this capability and restored luciferase expression to a level comparable with that measured with PR8 NS1 or empty pCAGGS plasmid.

Western blots confirmed that NS1 proteins were expressed, but to different levels (Fig. 1d), since the exogenously expressed H7N3 NS1 protein was prone to inhibit processing of its own mRNA. Despite the low level of expression, H7N3 NS1 was extremely potent at inhibiting luciferase expression, but this effect was abrogated by C-terminal truncation (Fig. 1c, d).

Taken together, our results suggests that while in the case of the PR8 NS1 protein the truncation does not affect dramatically the viruses ability to counteract IFN expression, in the context of the H7N3 NS1 it appears sufficient to trigger high IFN response in healthy cells and thus to enhance virus selectivity.

**NS1-77 truncation decreases H7N3 virus replication in IFN-competent non-malignant ductal pancreatic cells**

Replication of H7N3 and H7N3 NS1-77 viruses was monitored over a 72 h time course in IFN-deficient BxPC-3 and IFN-competent HPDE6 cells. In BxPC-3 cells, viral titres increased significantly between 24 and 72 h post-infection for both viruses and no significant difference was observed between the two viruses at any given time-point (Fig. 2a). Conversely, in IFN-competent HPDE6 cells, only the wild-type virus showed significant growth between 24 and 72 h post-infection, whereas the NS1-77 virus displayed poor replication (Fig. 2a).

Since the wild-type H7N3 virus appears able to counteract IFN expression in HPDE6 cells (Fig. 1b), the low replication observed in this cell line, suggests that some IFN-induced human genes likely contribute to limiting the replication of the avian isolate in non-malignant ductal cells. Thus, we performed targeted reverse transcription PCR (RT-PCR)
for type I IFN and associated genes in BxPC-3 and HPDE6 cells before and after infection with wild-type H7N3 virus (Fig. 2b). While some genes (e.g. RIG-1 and MAVS) were expressed and further induced after virus infection in both cell lines, others, such as IFN-α, IFN-β and MxA, were constitutively expressed at low levels and then induced upon infection only in HPDE6 cells. Conversely to many human IAVs, which are able to cope with Mx gene product, thanks to mutation within specific recognition sites on the NP protein, in general, avian IAVs are more sensitive to MxA activity and in order to be stably introduced in mammals often they have to acquire adaptive mutations to escape this restriction factor (Dittmann et al., 2008; Manz et al., 2013; Zimmermann et al., 2011). Because the H7N3 NP protein displays a typical avian signature (Rieger et al., 2015), it is possible that the expression of IFN-stimulated genes (ISGs), such as MxA, together with IFN, might account for the low growth rate and ensuing oncolytic activity (Kasloff et al., 2014) displayed by the wild-type H7N3 virus in HPDE6 cells.

The immunostimulatory activity of H7N3 NS1-77 virus in infected healthy cells enhances its oncolytic effect via IFN-mediated cell killing of neighbouring uninfected PDA cells

IFN-α and -β are multifunctional cytokines that have shown efficacy in the treatment in vitro and in vivo of several tumours types including PDAs (Booy et al., 2014; Lindner et al., 1997; Tomimaru et al., 2011; Vitale et al., 2006, 2007).

As the H7N3 NS1-77 virus induced high levels of IFN-β expression in IFN-competent HPDE6 cells, we hypothesised that by stimulating the antiviral response in bystander healthy cells, which in vivo would be in contact with the tumour, the truncated virus might indirectly trigger IFN-mediated killing of neighbouring uninfected PDA cells.

To test this hypothesis, confluent monolayers of IFN-competent HPDE6 cells were infected with H7N3 virus, H7N3 NS1-77 mutant virus or mock infected. After 24 h supernatants were collected and UV-treated to inactivate infectious particles in the suspension. IFN-β in the supernatants was assessed by ELISA and, as seen before, its expression following infection with NS1-77 mutant virus was significantly higher than for wild-type virus (Fig. 3a).

To evaluate whether the cytokines expressed from healthy cells could induce apoptosis in uninfected cancer cells, fresh monolayers of different PDA and HPDE6 cells were incubated with the conditioned media derived from the previous infection. After 16 h cells were assessed for apoptosis by the presence of cytoplasmic histone-associated DNA fragments (Duke & Cohen, 1986; Terui et al., 1995). Results (Fig. 3b) showed that the HPDE6 cell supernatants, containing IFN-β but not infectious virus, were capable of inducing apoptosis in various PDA cell lines to different extents. Indeed, diverse degrees of sensitivity to exogenous IFN-β, depending on the PDA cell line, have been previously reported (Booy et al., 2014; Vitale et al., 2007). Consistently with previous findings, BxPC-3 and AspC-1 cell lines displayed the highest sensitivity to conditioned media that contained IFN, followed by CFPAC-1, the more resistant Mia Paca-2 and finally by the non-malignant ductal pancreatic HPDE6 cells. In addition, the results showed that the HPDE6 cell supernatants derived from H7N3 NS1-77 infection provoked significantly higher levels of cell death in all the PDA cells than those derived from full length NS1 virus infection.

Next, to model a mixed population of cells, we tested the effect of infecting a monolayer containing a mix (ratio 1:1) of HPDE6 cells and a luciferase expressing BxPC-3 cell line derived from the original PDA cells, termed BxPC-3Luc. The mixture of HPDE6/BxPC-3Luc cells was infected with H7N3 or H7N3 NS1-77 viruses at different MOI in absence of exogenous trypsin and in the presence of FBS, which possesses proteases inhibitory activity (Schultzze et al., 1955), so viral replication was limited to a single cycle. The viruses ability to trigger BxPC-3Luc cell death in this experimental setting was evaluated through the decrease of luciferase signal compared to mock infection at 24 h post-infection.

While direct IAV infection of BxPC-3Luc cells should trigger cell death via direct lysis, we reasoned that infection of HPDE6 cells would additionally result in a high level of IFN-β production that, in a paracrine manner, would stimulate cell death in IFN-sensitive uninfected BxPC-3Luc cells (Fig. 4a).

A reduction of luciferase signal was detectable for both viruses at 24 h post-infection compared to mock infection and the extent of the decrease was overall greater at higher MOI (Fig. 4b). Moreover, at equal MOI, the amount of BxPC-3Luc cell death was higher for H7N3 NS1-77 virus infection than for the full length NS1 virus.

To confirm that PDA cell killing was at least partly due to the effect of virus infection of the HPDE6 cells, we repeated the experiment using BxPC-3Luc cells alone (Fig. 4c). Although virus infection still resulted in BxPC-3 cell death, there was no significant difference in the decrease of luciferase activity between the H7N3 and H7N3 NS1-77 viruses at any MOI used (Fig. 4d).

It may be noted that IFN produced by healthy cells might also act promoting an antiviral state, and thus inhibiting virus replication in PDA cells characterized by intact ISGs expression. However, besides BxPC-3, other PDA cell lines have been previously reported as defective or impaired in expression of specific ISGs even following stimulation with IFN (Moerdyk-Schauwecker et al., 2012). Thus, for this subset of PDA cells our results suggest that the NS1 truncated virus might have superior oncolytic effect compared to the wild-type virus in vivo, by adding to the direct virus-mediated lysis of infected PDA cells a powerful immunostimulatory effect on healthy cells, which can trigger killing of uninfected tumour cells.
H7N3 NS1-77 virus showed a beneficial effect in PDA xenograft mice model

To confirm the oncolytic activity of the modified virus in vivo, we tested the efficacy of each virus in the treatment of SCID (severe combined immunodeficiency) mice bearing BxPC-3Luc-derived solid tumours.

Equal numbers of BxPC-3Luc cells were injected subcutaneously into the right flank of SCID mice. After the mice developed palpable tumours, they were divided equally into three groups (n=5). A control group received intratumoural (IT) injections of PBS, whilst the other two groups received four 5\times10^4 p.f.u. (50 µl) IT injections of H7N3 or H7N3 NS1-77 viruses. The amount of virus used for each injection was almost 3 log10 lower than in trials previously performed for other tumour types using PR8 strain (Sturlan et al., 2010). Such a low virus dose was chosen to allow an appraisal of the contribution of both lytic and immunostimulatory activity of the viruses. The mice were monitored for signs of distress and tumour size was measured twice a week from the beginning of the treatment using a microcaliper. The mean percentage of volume increase compared to the beginning of the treatment (day 9) was calculated for each group at any given time point (Fig. 5a).

Despite the low amount of virus used for each injection, a reduction in tumour growth was observed in both groups treated with IAVs in comparison to the control group (PBS), with the H7N3 NS1-77 virus reaching the only significant beneficial effect (Fig. 5a). Bioluminescence imaging (BLI) performed at the end of the experiment showed that animals from the two groups treated with the H7N3 viruses presented tumours with different morphology. Indeed, while the majority of H7N3 treated mice were characterized by compact round-shaped tumours, the H7N3 NS1-77 treated animals presented mainly neoplasms with indented margins and discontinuity (Fig. 5b).

After all the mice were euthanized at 23 day post-implantation, five tumours per group were collected and assessed by real-time PCR for IAV Matrix (M) gene. All the tumours derived from mice injected with IAVs showed detectable virus RNA (Ct<35) (Fig. 5c), which suggests that viral replication within the tumour site was not different between the two viruses.

Because the two viruses were shown to replicate at similar levels in tumour BxPC-3 cells (Fig. 2a), the difference in the beneficial effect observed was unlikely ascribable to a higher viral replication. Thus, it might be due to the superior ability of the NS1-truncated virus to stimulate innate immune response from the healthy cells surrounding the tumour. Indeed, different publications confirmed that SCID mice, which lack T and B lymphocytes (Bosma et al., 1983), possess normal innate immune mechanisms including IFN-α/β expression that can reach levels similar to the responses of immunocompetent controls (Bray, 2001; Falk et al., 1995; Murphy et al., 2003).
Exchange of surface antigens does not affect the oncolytic potential of the NS1-77 avian influenza virus

Although our results confirmed that the H7N3 NS1-77 virus has restricted replication in IFN-competent human cells, the use of this avian isolate for cancer treatment might still raise some concern. Indeed, the H7N3 virus belongs to a subtype for which the human population is almost completely naïve and thus its use carries risk of ‘antigenic shift’ with circulating human strains, within a treated patient, that might result in a new potentially pandemic virus (Neumann et al., 2009). This event would require coinfection of the same cell by the two viruses within a hospitalized patient. However, the fact that the pancreas is not considered a typical replication site for IAVs in humans, the modest replication level displayed by the H7N3 NS1-77 virus in mammalian non-malignant cells together with its administration in a confined space should enhance the safety of the potential treatment. Furthermore, rapid accumulation of virus-neutralizing antibodies in recipients has

Fig. 4. Immunostimulatory activity of H7N3 viruses. (a) Proposed mechanism of immunostimulation in a mixed monolayer of non-malignant pancreatic ductal HPDE6 cells and tumour IFN-deficient BxPC-3Luc cells stably expressing the luciferase gene. In addition to triggering direct lysis of infected BxPC-3Luc cells, IAV stimulates IFN production from HPDE6 cells, which in turn can promote cell death of uninfected IFN-sensitive PDA cells enhancing the overall oncolytic activity of the virus. Apoptotic bodies, dying cells; protease activated virus, input virus; inactive virus, virus arising from infected cells in presence of FBS. (b) Luciferase activity in cell lysates of mixed monolayers of HPDE6 and BxPC-3Luc cells after 24 h infection with H7N3 and H7N3 NS1-77 viruses at different MOI or following mock infection. The luciferase signal is expressed in RLU. (c) Proposed mechanism of cell killing by IAV in monocultures of tumour BxPC-3 cells. Since BxPC-3 cells lack IFN expression, cell death will be exclusively the result of the direct virus-mediated lysis of infected cells. (d) Luciferase activity in cell lysates from monocultures of BxPC-3Luc cells alone at 24 h post-infection with H7N3 and H7N3 NS1-77 viruses using different MOI or following mock infection. The luciferase signal (RLU) provides a direct measurement of BxPC-3Luc cell viability. All data displayed in panel B and D represent means±s of representative experiments (n=4 for each MOI). Statistical significance was determined using two-way ANOVA followed by Tukey’s multiple comparison test.
Fig. 5. Efficacy of H7N3 and H7N3 NS1-77 virus treatment in SCID mice bearing human BxPC-3Luc-derived tumours. (a) After solid tumours were established (day 9), animals were randomly divided into three groups (n=5), which were treated four times with $5 \times 10^4$ p.f.u. of H7N3, H7N3 NS1-77 viruses or with PBS. Tumour sizes were monitored by caliper measurements and the percentage of volume increase was calculated as described in Methods. Comparison of groups was performed by using two-way ANOVA followed by the Tukey’s multiple comparison test. (b) Bioluminescence imaging (BLI) of BxPC-3Luc solid tumours from animals belonging to H7N3 and H7N3 NS1-77 treatment groups at 23 days post-implantation. (c) Ct (cycle threshold) results for real-time RT-PCR targeting the influenza virus Matrix gene (M) in tumour homogenates from mice treated with H7N3, H7N3 NS1-77 or PBS.
been reported also in the case of treatment with viruses that normally do not infect humans (Tesfay et al., 2014; Zamarin & Palese, 2012), and indeed one of the biggest challenges for OVs consists in eluding the adaptive immunity that might undermine their efficacy especially in a multi-dose regimen (Miest et al., 2011; Russell et al., 2012).

Nonetheless, the number of IAV serotypes available together with the segmented nature of the influenza genome facilitates subtype exchange, allowing the generation of a suite of similar viruses that differ only in surface antigens, and thus offering an opportunity to address either safety or pre-existing immunity issues. To test the feasibility of this approach, we engineered a new H7N3 NS1-77 virus in which the genes expressing the surface antigens haemagglutinin (HA) and neuraminidase (NA) were replaced with those of the well characterized human strain H1N1 A/ Puerto Rico/8/1934 (PR8). In vitro experiments performed using this 6:2 recombinant virus, termed PR8 H1/N1-2962 NS1-77, confirmed that the exchange of surface antigens did not alter the virus’ ability to trigger apoptosis in BxPC-3 cells at levels similar to the parental strains and thus, in line with previous data (Kasloff et al., 2014), at higher levels compared to the H1N1 PR8 virus (Fig. 6a). Therefore, we conclude that the marked ability of the avian isolate to trigger PDA cell death does not depend on properties determined by the HA gene such as sialic acid receptor binding or fusion, but rather on the activity of its internal pro-apoptotic genes. In addition, the new surface glycoproteins did not affect the immunostimulatory effect previously displayed by the H7N3 NS1-77 virus (Fig. 6b) in non-malignant pancreatic ductal HPDE6 cells, which might be at the basis of the lower levels of apoptosis induced in this cell line by the two deletant viruses in comparison to the wild-type H7N3 virus (Fig. 6a).

Taken together, our results highlights the possibility to further improve the safety of the treatment and/or to modulate the sensitivity to pre-existing immunity by changing the virus’ surface glycoproteins without affecting its apoptotic skills.

**Conclusion**

In the present study, for the first time to our knowledge, the oncolytic potential of an engineered avian-origin IAV was evaluated against PDA cells. Indeed, no recombinant IAV of any origin has been appraised so far for PDA treatment, and the relative small number of previous investigations concerning the use of IAV for virotherapy were focused on the human strain H1N1 A/Puerto Rico/8/34 (Bergmann et al., 2001; Muster et al., 2004; Sturlan et al., 2010; Wolschek et al., 2011). Importantly, the choice of a particular viral isolate is likely to affect its efficacy as an OV because IAVs differ in their ability to counteract host IFN response (Geiss et al., 2002; Hayman et al., 2006; Kochs et al., 2007), induce apoptosis (Kasloff et al., 2014) and in their sensitivity to host ISGs (Dittmann et al., 2008).

Engineered NS1 deletions or truncations previously described in the literature have mostly been aimed at the development of novel attenuated influenza vaccines (Ngunjiri et al., 2015; Pica et al., 2012; Wacheck et al., 2010). Previous studies developing the human H1N1 PR8 strain as an OV also utilized NS1 deletion or truncation to improve the selectivity of the virus to replicate in IFN-deficient tumour cells (Muster et al., 2004; Sturlan et al., 2010). However, none of these studies had described the bystander effect that we directly tested here, whereby H7N3 NS1 truncation significantly enhanced the killing of uninfected PDA cells through stimulation of IFN expression from the

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**Fig. 6.** Exchanging surface antigens does not affect apoptosis induction or immunostimulatory ability. (a) Immunoassay detection of cytoplasmic histone-associated DNA fragments in BxPC-3 and HPDE6 cells at 16 h post-infection with H7N3, H7N3 NS1-77, PR8 H1/N1-2962 NS1-77 and H1N1 PR8 viruses (MOI=1). Cell death shown as absorbance sample/absorbance mock+SD of one representative experiment (n=2). Statistical significance versus wild-type H7N3 virus was tested using two-way ANOVA followed by Dunnett’s multiple comparison test. (b) IFN-β ELISA results for supernatants derived from BxPC-3 and HPDE6 cells infected with H7N3, H7N3 NS1-77, PR8 H1/N1-2962 NS1-77 viruses or mock infected. For each group raw absorbance values at 450 nm are represented on the left y axis and the IFN-β concentrations (pg ml⁻¹) obtained by comparison with a standard curve are shown on the right y axis. All values represent means+SD of one representative experiment (n=2).
healthy cells. We postulate that increased IFN-α levels in the tumour microenvironment after infection with NS1-77 virus might also contribute to an inhibition of angiogenesis as previously reported (Indraccolo, 2010; von Marschall et al., 2003), and this could be directly tested in future studies.

The data presented also suggest that the specific apoptotic ability for which the H7N3 virus was selected resides in its internal pro-apoptotic genes and that this feature might be exploited for serotype exchange in order to tackle safety issues or to avoid pre-existing immunity. Although immune-deficient animal models are suitable for the evaluation of effective replication in and destruction of human tumours by OVs, they cannot assess the complete safety and efficacy profile of the virus in normal tissue, nor do they permit evaluation of the impact of adaptive immunity on overall virus potency. Thus, further in vivo trials using immunocompetent or humanized mouse models will be required to test the PR8 H1/N1-2962 NS1-77 virus oncolytic activity and the possibility to exploit antigenic exchange for PDA treatment.

In conclusion, our work highlights the importance of strain specific characteristics in the design of an influenza-based OV. Although the evaluation of IFN production and sensitivity of PDA subset should be used to predict responsiveness to the treatment, we demonstrate improvement in the selectivity and tumour debulking activity of an avian IAV isolate, primarily chosen on the basis of its apoptotic potency in PDA cells, by coupling the direct viral lysis with a stronger activation of the innate immune response.

**METHODS**

**Cells.** Human embryonic kidney cells (293T) and PDA cell line Mia Paca-2 were grown in Dulbecco’s modification of Eagle’s medium (DMEM, Gibco) supplemented with 10% FBS (Biosera), 1% MEM non-essential amino acid solution (NEAA, Sigma) and 1% penicillin-streptomycin (P/S, Gibco). Geneticin (G-418, Gibco) was added at the concentration of 0.5 mg ml$^{-1}$ in the case of Npro-MDCK cells, which express NPro gene of bovine viral diarrhoea virus (BVDV) and thus lack of IFN expression, and for A549-IFNLuc cells, which contain a stable integrate firefly luciferase gene driven by the IFN-β promoter. The immortalized epithelial cell line from normal human pancreatic ducts HPDE6 (Furukawa et al., 1996; Ouyang et al., 2000) and the PDA cell lines AsPC-1 and BxPC-3 were maintained in supplemented RPMI 1640 medium (Gibco), while Iscove’s medium (Gibco) was used to grow CFPAC-1 cells.

BxPC-3 cells stably expressing luciferase gene (BxPC-3-Luc) were generated using lentivector transduction. Briefly, semi-confluent monolayers of 293T cells were co-transfected in 10 cm$^2$ Petri dishes with HIV gag/pol construct, vesicular stomatitis virus G protein (VSV-G) construct and psiR-ﬁre ﬂuor luciferase reporter construct using Lipofectamine 2000 (Invitrogen). Supernatant was harvested 48 h post-transfection, ﬁltered through 0.45 μm ﬁlters and stored at ~8°C. Transduction was performed in a 12-well plate by adding 500 μl well$^{-1}$ of lentivirus suspension in serum-free DMEM on BxPC-3 cells. After 24 h incubation at 37°C, the medium was replaced with fresh medium containing 3% FBS DMEM and cells were further incubated for 72 h. The lentivector transduction efficiency was assessed by monitoring expression of luciferase in the target cells using the Luciferase Assay Reporter System (Promega).

**Viruses.** cDNA copies of the H7N3 A/turkey/Italy/2962/03 and H1N1 A/Puerto Rico/8/34 (PR8) viral RNA segments were inserted into RF483 vector, derived from the pHW2000 plasmid previously generated by Hoffmann et al. (2000), using the In-Fusion Dry-Down PCR Cloning Kit (Clontech). Plasmids obtained were propagated in One Shot TOP10 Chemically Competent E. coli (Invitrogen), purified by EndoFree Plasmid Maxi Kit (Qiagen) and confirmed by sequencing. Mutations of interest in the NS1 genes (segment 8) were introduced by PCR using QuickChange Site-Directed Mutagenesis Kit (Stratagene) as follows. Using the set of primers H7N3/NS1-77fw and H7N3/NS1-77rv (Table 1), two stop codons (AAATAG) were generated by two site mutations (A232T, A235T, A236A) after amino acid 77 in the H7N3 NS1 ORF. A single nucleotide mutation A232T was introduced in segment 8 of H1N1 PR8 using PR8/NS1-77fw and PR8/NS1-77rv primers (Table 1) in order to create a stop codon (TAA) in the NS1 ORF after 77 aa.

The virus rescue method used was adapted from Neumann et al. (1999) as previously described by Elleman & Barclay (2004). To generate recombinant NS1-77 viruses, plasmids contained the mutated segment 8 RNAs were substituted in place of those for the equivalents wild-type segments. The PR8 H1/N1-2962 NS1-77 virus was obtained by co-transfecting the plasmids expressing the surface antigens HA and NA of the H1N1 PR8 virus together with the six plasmids expressing H7N3 NS1-77 viruses internal genes. Viral titres were determined by plaque assay on MDCK-Npro cells (Matrosovich et al., 2006).

**Generation of pCAGGS/NS1 constructs.** pCAGGS/H7N3 NS1 expression construct was generated as previously described by Hayman et al. (2006). Briefly, PCR mutagenesis was used to remove the splice acceptor site (AG) from the H7N3 A/turkey/Italy/2962/03 NS1 coding sequence. In PCR1, the primer pairs H7N3/NS1NotIF and SAMR (Table 1) were used, producing a ~550 bp ampiclon. PCR2 included primers SAMF and H7N3/NS1MluIR (Table 1) and resulted in a ~210 bp product. PCR1 and PCR2 were performed starting from the viral RNA as template using QiAGEN OneStep RT-PCR Kit (Qiagen). A third overlapping PCR3 was then carried out using the NS1NotIF and NS1MluIR primers. The 720 bp PCR3 product was then digested with NotI and MluI restriction enzymes (New England BioLabs) and ligated into pCAGGS vector, which allow the fusion of the expressed protein with a 14 aa long simian virus 5 epitope (V5 tag) at its carboxyl terminus. The construct was then propagated in One Shot TOP10 Chemically Competent E. coli (Invitrogen), purified by EndoFree Plasmid Maxi Kit (Qiagen) and confirmed by sequencing. A second construct pCAGGS/H7N3 NS1-77, encoding the first 77 aa of the NS1 protein from H7N3 A/turkey/Italy/2962/03, was generated by PCR using the forward primer H7N3/NS1NotIF and the reverse primer H7N3/NS1-77MluIR (Table 1). A pCAGGS/PR8 NS1 construct containing the full length NS1 from H1N1/PR8/8/34 modified to remove the splice acceptor site was previously generated in our laboratory (Hayman et al., 2006).

**A549-IFNLuc assay.** A549-IFNLuc cells were incubated at 37°C in a 24-well plate with different reverse genetics viruses diluted in serum-free DMEM (MOI=1). After 1 h absorption, the input viruses were replaced with 3% FBS DMEM and then cells were further incubated for 8 h. Cells were washed once with PBS before being lysed with 100 μl well$^{-1}$ of Passive Lysis Buffer (Promega) rocking the plate for 15 min at room temperature and then frozen and thawed for three times at ~80°C. Luciferase activity was measured in a luminometer (FLUOSTar OPTIMA, BMG Labtech) using the Luciferase Assay System (Promega).

**ISG54 luciferase assay.** HPDE6 and BxPC-3 cells were either mock infected or infected with H7N3 or PR8 recombinant viruses bearing full length or truncated NS1 protein (MOI=1) in serum-free DMEM in 24-

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Table 1. List of primers used for the generation of the different NS1 constructs in the present study

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1N1 A/Puerto Rico/8/34</td>
<td>PR8/NS1-77fw</td>
<td>5'-AACCCGATGGGACCTTAAATGACATGCGG-3'</td>
</tr>
<tr>
<td></td>
<td>PR8/NS1-77rv</td>
<td>5'-AGGGCCATGATCATTAAAGTCCTCAGTCGATAGT3'-3'</td>
</tr>
<tr>
<td>H7N3 A/turkey/Italy/2962/03</td>
<td>H7N3/NS1-77fw</td>
<td>5'-GGAGGAAGATCTGAGGGACCTTTAATAGACTATTACCTTACGTCGGC-3'</td>
</tr>
<tr>
<td></td>
<td>H7N3/NS1-77rv</td>
<td>5'-CCGGCATTGAGTTAATAGCTTATTAAGTGGCCTCATCGACTTCTCTCC-3'</td>
</tr>
<tr>
<td></td>
<td>H7N3/NS1NotIF</td>
<td>5'-TATGGGCCGCCAGACACACAGGGCTGACAACAAACA-3'</td>
</tr>
<tr>
<td></td>
<td>SAMR</td>
<td>5'-GCGCTACGATGCTTGCGAAGAGAAGGTATAGGG-3'</td>
</tr>
<tr>
<td></td>
<td>SAMF</td>
<td>5'-CCTTACCTTCTTCTCCGGACATCATGATAGAGA-3'</td>
</tr>
<tr>
<td></td>
<td>H7N3/NS1MuIR</td>
<td>5'-GGAGAGCGGTAACCTCTGACTCAATGTTCTCGGCA-3'</td>
</tr>
<tr>
<td></td>
<td>H7N3/NS1-77MuIR</td>
<td>5'-GGAGAGCGGTAAGTGCCCTCATCACTTCTCTCC-3'</td>
</tr>
</tbody>
</table>

well plates. After 1 h adsorption, virus suspensions were replaced with 3% FBS DMEM and cells were incubated for 8 h. The supernatants were then collected and exposed on ice to the ultraviolet radiation from a germicidal lamp for 5 min in order to inactivate any possible viral particle before being stored at −80 °C. Plasmids ICSG4Luc, encoding the firefly luciferase gene driven by the inducible promoter ICSG4, and RenillaLuc, encoding the Renilla luciferase, were co-transfected into 293T cells using Lipofectamine 2000 (Roche). At 8 h post-transfection, the 293T medium was replaced with the supernatants previously inactivated and cells were further incubated at 37 °C for 24 h. Next, 293T cells were washed once with PBS and then lysed with 100 µl well −1 of Passive Lysis Buffer (Promega) rocking the plate for 15 min at room temperature before freezing and thawing them for three times at −80 °C. The firefly and the Renilla luciferase activities were measured in a luminesimeter (FLUOstar OPTIMA, BMG Labtech) using the Dual-Luciferase Reporter Assay System (Promega).

Post-transcriptional limitation of host gene expression. 293T cells were co-transfected with pCAGGSLuc plasmid, which constitutively expresses firefly luciferase, and pCAGGS/PR8 NS1, pCAGGS/H7N3 NS1, pCAGGS/H7N3 NS1-77 or empty pCAGGS (K-) plasmid. After 8 h incubation at 37 °C, cells were lysed in Passive Lysis Buffer (Promega), rocking the plate for 15 min at room temperature. In order to assure the maximum release of the luciferase proteins cells were frozen and thawed for 3 times at −80 °C. The firefly luciferase activity was measured in a luminesimeter (FLUOstar OPTIMA - BMG Labtech) using the Luciferase Reporter Assay System (Promega).

Western blot. Cells lysates were treated in dissociation buffer for 5 min at 96 °C and then separated by electrophoresis in 12% polyacrylamide gels. Proteins were transferred by electrophoblotting onto immunoblot Hybond ECL membranes (GE Healthcare), which were washed with PBS and then blocked overnight at 4 °C with PBS and 5% skimmed milk (Bio-Rad). After further washing with PBS, the membranes were incubated for 1 h at room temperature with PBS solution containing 0.05% Tween-20 (Sigma), 5% skimmed milk (Bio-Rad) and mouse ANTI-V5 tag antibody (Abd Serotec). Next, the membranes were washed as previously described and then incubated for 1 h at room temperature with goat anti-mouse IgG horseradish peroxidase linked whole antibody (GE Healthcare). Vinculin was detected as loading controls using goat anti-vinculin (N-19) antibody (Santa Cruz Biotechnology) followed by incubation with donkey anti-goat IgG-HRP antibody (Santa Cruz Biotechnology). Visualization of protein bands in Amersham Hyperfilm ECL (GE Healthcare) was performed using ECL Plus Western Blotting Detection System (GE Healthcare).

Virus replication kinetics in pancreatic cell lines. Replication of H7N3 NS1-77 versus H7N3 virus in HPDE6 and BxPC-3 cells was monitored over a 72 h time course. Cells were seeded in a 24-well plate and then infected with an MOI of 0.001. After 1 h adsorption, the inoculum was removed and replaced with 500 µl well −1 of serum-free media containing 1% Pen/Strep solution and 0.1 µg ml −1 of TPCK-trypsin (Sigma). At 24, 48 and 72 h post-infection cell suspensions were harvested and centrifuged, then the supernatants were stored at −80 °C. Viral titers were determined for all the samples within the same plaque assay session on MDCK-Npro cells (Matrosovich et al., 2006).

Expression of IFN-related genes in BxPC-3 and HPDE6 cells. Cells were either mock-treated or infected with H7N3 A/turkey/Italy/2962/03 (MOI=0.1). After 16 h total RNA was extracted from the cells using Nucleospin RNA II kit (Macherey-Nagel) and amplified by RT-PCR using OneStep RT-PCR Kit (Qiagen). PCR was carried with the following conditions: reverse transcription at 50 °C for 30 min, denaturation at 94 °C for 15 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s, extension at 72 °C for 45 s before a final elongation step at 72 °C for 8 min. All the primers used for the PCR were previously published and designed to amplify cellular mRNAs by Moerdyck-Schauwecker et al. (2012). PCR products were electrophoresed on a 1% agarose gel with GelRed nucleic acid stain (Biotium) and pictures acquired using Gel Doc EZ Imager (Bio-Rad).

Quantitative measurement of IFN-β and apoptosis. Quantitative determinations of IFN-β in cell supernatants and cytoplasmic histone-associated DNA fragments in human pancreatic cells were performed using the VeriKine™ Human Interferon Beta ELISA Kit (pbl Assay Science) and the Cell Death Detection ELISAPLUS Kit (Roche), respectively.

Evaluation of virus immunostimulatory activity. Confluent monolayers of IFN-competent HPDE6 cells were infected in 12-well plates with H7N3, H7N3 NS1-77 viruses (MOI=1) or mock infected. After 1 h adsorption at 37 °C, the media were replaced with RPMI 1640 supplemented with 3% FBS, 1% Pen/Strep and 1% NEAA and cells were further incubated for 24 h. Supernatants were then collected and exposed for 10 min under germicidal lamp to inactivate potential infectious particles in the suspension as confirmed by subsequent plaque assay. The VeriKine Human Interferon Beta ELISA Kit (pbl Assay Science) was used for quantitative measurement of IFN-β in the supernatants. Next, different monolayers of PDA and HPDE6 cells in 24-well plates were incubated with the conditioned media derived from the previous infection. After 16 h incubation, the media were discharged and cells were washed with PBS and tested for the presence of cytoplasmic histone-
associated DNA fragments using the Cell Death Detection ELISAPLUS photometric enzyme immunoassay (Roche – Life Science).

Confluent mixture of HPDE6-BxPC-3-Luc cells or BxPC-3-Luc cells alone were grown in 96-well plates. Cells were then washed three times with PBS and infected with H7N3 or H7N3 NS1-77 viruses at MOI=0.1, 0.01, 1 or mock infected. After 1 h adsorption, cells were supplemented with RPMI 1640 with 0.5% FBS, 1% Pen/Strep, 1% NEAA and further incubated at 37 °C until 24 h post-infection when they were washed with PBS and lysed in Passive Lysis Buffer (Promega). Next, 5 µl of cell lysates were combined with 100 µl of BrightGlow buffer and substrate mixture (Bright-Glo Luciferase Assay System, Promega). Luciferase signal was read within 5 min in GloMax-Multi Detection System (Promega).

In vivo tumour model. The in vivo experimental protocol was investigated and approved by the Italian Ministry of Health (protocol 130/2011). Six weeks old pathogen-free SCID-B17 female mice were maintained in laminar flow racks and microisolators cages under pathogen-free conditions and received autoclaved food and water ad libitum. Mice were inoculated subcutaneously in the right flank with 100 µl of 5 × 10^6 BxPC-3-Luc cells. Palpable tumours developed after 9 days and mice were then randomly divided into three different groups (n=5 per group) receiving an intra-tumoural (IT) inoculation of 5 × 10^5 p.f.u. (50 µl) of H7N3 A/turkey/Italy/2962/03, H7N3 NS1-77 viruses or PBS (negative control). The procedure was subsequently repeated 3, 7 and 10 days later for a total of four inoculations per treatment group. The overall physical condition and behaviour of the mice were monitored daily, and caliper measurements of tumour size were performed at regular intervals following initial injection. Tumour volumes were determined according to the formula V= (L × W^2) / 2 (V, volume; L, length; W, width). The percentage of volume increase from the beginning of the treatment was calculated as follows: Volume increase (%) = (Vt – V0)/(V0 × 100). Tumour morphology and presence of metastasis were monitored by BLI at the end of the experiment (day 23). Following post-mortem examination, tumours were collected, snap-frozen and tested for viral RNA presence by real-time RT-PCR using primers and probes targeting the conserved Matrix (M) gene of type A influenza virus (Spackman et al., 2002).

Statistical analysis. Analysis of the data was performed using GraphPad Prism 6 for Windows (GraphPad Software Inc., La Jolla, CA). Symbol and meaning: *, P<0.05; **, P<0.01; ****, P<0.001; *****, P<0.0001.

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Engineering an avian influenza virus for PDA virotherapy


