ORIGINAL ARTICLE

Synergistic cytotoxicity of radiation and oncolytic Lister strain vaccinia in ^{V600D/E}BRAF mutant melanoma depends on JNK and TNF- α signalingJN Kyula¹, AA Khan¹, D Mansfield¹, EM Karapanagiotou¹, M McLaughlin¹, V Roulstone¹, S Zaidi¹, T Pencavel¹, Y Touchefeu¹, R Seth¹, NG Chen², YA Yu², Q Zhang², AA Melcher³, RG Vile^{3,4}, HS Pandha⁵, M Ajaz⁵, AA Szalay² and KJ Harrington¹

Melanoma is an aggressive skin cancer that carries an extremely poor prognosis when local invasion, nodal spread or systemic metastasis has occurred. Recent advances in melanoma biology have revealed that RAS-RAF-MEK-ERK signaling has a pivotal role in governing disease progression and treatment resistance. Proof-of-concept clinical studies have shown that direct BRAF inhibition yields impressive responses in advanced disease but these are short-lived as treatment resistance rapidly emerges. Therefore, there is a pressing need to develop new targeted strategies for BRAF mutant melanoma. As such, oncolytic viruses represent a promising cancer-specific approach with significant activity in melanoma. This study investigated interactions between genetically-modified vaccinia virus (GLV-1h68) and radiotherapy in melanoma cell lines with BRAF mutant, Ras mutant or wild-type genotype. Preclinical studies revealed that GLV-1h68 combined with radiotherapy significantly increased cytotoxicity and apoptosis relative to either single agent in ^{V600D}BRAF/^{V600E}BRAF mutant melanoma *in vitro* and *in vivo*. The mechanism of enhanced cytotoxicity with GLV-1h68/radiation (RT) was independent of viral replication and due to attenuation of JNK, p38 and ERK MAPK phosphorylation specifically in BRAF mutant cells. Further studies showed that JNK pathway inhibition sensitized BRAF mutant cells to GLV-1h68-mediated cell death, mimicking the effect of RT. GLV-1h68 infection activated MAPK signaling in ^{V600D}BRAF/^{V600E}BRAF mutant cell lines and this was associated with TNF- α secretion which, in turn, provided a prosurvival signal. Combination GLV-1h68/RT (or GLV-1h68/JNK inhibition) caused abrogation of TNF- α secretion. These data provide a strong rationale for combining GLV-1h68 with irradiation in ^{V600D/E}BRAF mutant tumors.

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INTRODUCTION

Oncolytic viruses have the remarkable biological property of being able to replicate selectively in cancer cells, thus mediating tumor-selective cytotoxicity. A number of agents from a diverse range of viral families are currently in preclinical or clinical development. Indeed, herpes simplex virus type 1 (OncoVEX^{GM-CSF}), reovirus (Reolysin) and vaccinia virus (JX-594) have all reached late-stage, randomised clinical trials either as single agent therapies or in combination with cytotoxic chemotherapy. In addition, recent studies have highlighted the fact that combining oncolytic virotherapy with ionizing radiation (RT) may lead to synergistic antitumor efficacy and translational phase I/II clinical trials have been completed.^{1,2} However, the reported complex effects of RT on viral infectivity, replication, gene expression and cytotoxicity mean that detailed mechanistic preclinical studies are an essential prerequisite to trials of new oncolytic viral agents in combination with RT.

Vaccinia virus strains are promising candidates for clinical application in treating cancer. They show a preference for replication in dividing cells and, indeed, are able to manipulate signaling through the mitogen-activated protein kinase (MAPK) pathway to create a cellular environment that is conducive to viral

replication.^{3,4} Tumor cell lines with constitutive activation of the MAPK pathway are likely to be susceptible to vaccinia virus-mediated oncolysis. In addition, ionizing RT can activate MAPK signaling and this may further increase viral replication in infected and irradiated tumor cells.⁵

A Lister strain oncolytic vaccinia virus (GL-ONC1, Genelux Corporation, San Diego, CA, USA) that has been attenuated by insertion of β -galactosidase, β -glucuronidase and *Renilla* luciferase-green fluorescent protein expression cassettes into the thymidine kinase (J2R), hemagglutinin (A56R) and F14.5L loci is currently in phase I clinical evaluation. The antitumor activity of the equivalent, non-clinical grade virus (GLV-1h68) has been profiled in preclinical studies as part of the planning process for subsequent phase I and II clinical trials.^{6–12} These studies have included a detailed analysis of the combination of GLV-1h68 with RT in malignant melanoma. This tumor type frequently presents with multiple cutaneous and subcutaneous deposits and represents an attractive model for early phase clinical studies of combinations of oncolytic viruses and RT—not least because of its poor prognosis and relative radioresistance, but due to its accessibility for direct intratumoral injection and the potential to obtain repeated biopsies to measure pharmacodynamic

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markers.¹³ In addition, recent impressive results with the BRAF inhibitor, vemurafenib, suggest that malignant melanoma is an excellent scenario in which to test molecularly targeted agents.¹⁴

In this report, we show that oncolytic vaccinia virus mediates synergistic toxicity with RT in malignant melanoma, but only those that harbor ^{V600D}BRAF and ^{V600E}BRAF mutations. These data provide a strong rationale for testing this approach in other ^{V600D}BRAF or ^{V600E}BRAF mutant tumor types (for example, colorectal and thyroid cancers) and support the clinical translation of this strategy.

RESULTS

GLV-1h68 and RT exert synergistic cytotoxicity in ^{V600E}BRAF mutant melanoma

The effect of GLV-1h68 infection in a panel of melanoma cell lines with wild-type BRAF/RAS, ^{V600D/E}BRAF mutation or RAS (K- and N-) mutation was assessed 72 h post-infection by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Supplementary Figure S1 and Supplementary Table S1). All melanoma cell lines were found to be sensitive to GLV-1h68, with the MeWo and PMWK wild-type cell lines being the most sensitive to GLV-1h68 with IC₅₀ values of 0.5 MOI (multiplicities of infection) and 0.05 MOI, respectively, by formal quantitative colorimetric assay. The WM17971 (K-RAS mutant) and D04 (N-RAS mutant) cell lines both had IC₅₀ values of 1 MOI. The A375 ^{V600E}BRAF and WM266.4 ^{V600D}BRAF mutant cell lines were found to be less sensitive to GLV-1h68 with IC₅₀ values of 2.5 MOI. We extended the panel of ^{V600E}BRAF mutant cell lines to include SKMel28 and Mel624, which both had IC₅₀ values of 2.

We next determined the effect of combining GLV-1h68 and RT. Cells were irradiated to 1, 3 or 5 Gy fractions and 6 h later were infected with GLV-1h68 at MOI ranging from 0.1 to 10 in 96-well plates for MTT assay (Figure 1a), or to a single fraction of 5 Gy and 6 h later infected with GLV-1h68 at MOI between 0.001–1.0 in 24-well plates for SRB assay (Figure 1b) and crystal violet staining (Supplementary Figure S2A). Cell survival was determined 72 h post-infection. The effect of RT alone did not cause a significant increase in cell death by MTT in all cell lines. There was a slight decrease in the A375 and WM266.4 BRAF mutant cell lines following RT at 5 Gy as shown in the SRB assay, but this was seen to be non-significant (Figure 1c). We found significantly greater cytotoxicity following combined GLV-1h68 and RT in the ^{V600E}BRAF and ^{V600D}BRAF mutant cell lines, A375 and WM266.4, respectively, by MTT (Figure 1a). To evaluate the level of synergy between RT and GLV-1h68, we used Bliss independence analysis as previously described.^{15–18} This methodology uses the formulae $E_{IND} = E_A + E_B - E_A \times E_B$ and $\Delta E = E_{OBS} - E_{IND}$, where: E_A and E_B are the fractional effects of factors A and B, respectively; E_{IND} is the expected effect of an independent combination of factors; E_{OBS} is the observed effect of the combination. If ΔE and its 95% confidence interval (CI) were >0, then synergy was observed. If ΔE and its 95% CI were <0, then antagonism was observed. If ΔE and its 95% CI were 0, then the combination was independent. The results from Bliss analysis demonstrated a strong synergistic effect between GLV-1h68 and RT in the ^{V600E}BRAF and ^{V600D}BRAF cell lines, where ΔE was >1 (Figure, 1b). There was, however, no synergistic effect in the MeWo and PMWK wild-type cell lines or the WM17971 (K-RAS mutant) and D04 (N-RAS mutant) cell lines. The SRB analysis showed enhanced cell kill in the BRAF mutant A375 and WM266.4 cell lines where P -values were between 0.01–<0.001 (Figure 1c). This was further confirmed in the crystal violet assay (Supplementary Figure S2), which showed no increase in cell kill with RT alone. All melanoma cell lines showed an increase in cell kill with increasing doses of GLV-1h68; of note, the wild-type MeWo and PMWK cell lines formed bigger plaques with increasing GLV-1h68 infection. While the A375 and WM266.4 BRAF mutant cell lines as well as the K-RAS WM1791 cells appeared more sensitive. Furthermore, the SRB assay quantitatively showed that all cell lines

responded to increase in cell kill when treated with GLV-1h68, but the PMWK wild-type cell remained the most sensitive (Figure 1c). More importantly, the quantitative SRB assay showed a significant increase in cell kill when RT and GLV-1h68 were combined in the A375 and WM266.4 BRAF mutant cell line (Figure 1c).

In addition, colony formation assays were carried out to assess RT and GLV-1h68 interaction. MeWo, A375 and D04 cells were exposed to a single fraction of 2 or 5 Gy and surviving fraction determined. This was to assess the dose to take forward for combining RT and GLV-1h68. We found that all three cell lines had a very similar surviving fraction at 2 and 5 Gy (Figure 1d and Supplementary Figure S2B). In view of this, we used 2 Gy of RT in combination with GLV-1h68 at an MOI of 0.01. Colony formation assays confirmed an increase in cell death due to combined virus and RT therapy in ^{V600E}BRAF mutant cells (Figure 1e). Furthermore, the other two ^{V600E}BRAF mutant cell lines, Mel624 and SKMel28, showed enhanced cytotoxicity when assayed for MTT, crystal violet and SRB (Supplementary Figure S3). However, no significant increase in cytotoxicity was observed in the wild-type MeWo and PMWK or RAS mutant D04 (N-RAS) and WM17971 (K-RAS) cell lines (Figures 1a–c and e).

Combined GLV-1h68 and RT enhances tumor growth delay *in vivo* and prolongs survival in A375 ^{V600E}BRAF mutant xenograft tumors

Subsequently, we assessed the *in vivo* relevance of the observations in Figure 1 in A375 xenograft tumors. BALB CDI nude mice received subcutaneous injections of 5×10^6 A375 cells on the right flank. Once xenograft tumors had reached ~5 mm in diameter, the mice were randomly allocated into groups of 14 and treated with PBS alone, GLV-1h68 alone at 1×10^4 plaque forming units (PFU), RT alone (6 Gy in three fractions) or a combination of 6 Gy in three fractions plus 1×10^4 PFU GLV-1h68. When RT and GLV-1h68 were combined, the virus was given after the second fraction of RT. GLV-1h68 and PBS were given by intratumoral injection. The treatment regimen is summarized in Figure 2a. Four mice from each treatment group were culled after the end of the treatment on day 7 and tumors lysed and assessed for apoptosis by caspase 3 cleavage and TUNEL assay. Tumor size from the remaining 10 mice from each group was measured by Vernier callipers and volumes were plotted as soon as treatment began, as shown in Figure 2b. Tumors in all groups were slow to propagate until day 30, after which point, a loss of growth delay was observed in control, RT and virus alone groups. Combined treatment with GLV-1h68 and RT attenuated tumor growth compared with either RT or GLV-1h68 alone. We performed multiple Mann–Whitney U -tests at serial time points after day 45, which demonstrated that statistically significant differences between groups persisted until the end of the experiment (Figure 2c). In addition, survival analysis showed that mice treated with a combination of GLV-1h68 and RT took a significantly longer time to reach the experiment endpoint (tumor > 15 mm diameter) than mice treated with either GLV-1h68 or RT alone, with a P -value of 0.0099 (Figure 2d). Consistent with the tumor growth data, western blotting analysis showed an increase in caspase 3 cleavage when RT and GLV-1h68 were combined (Figure 2e). Furthermore, TUNEL assay confirmed enhanced apoptosis with the combined RT and GLV-1h68 compared with GLV-1h68 or RT treatment alone (Figure 2f).

RT does not enhance GLV-1h68 viral replication in ^{V600D/E}BRAF mutant melanoma cells

To investigate whether the observed synergy between RT and GLV-1h68 in ^{V600D/E}BRAF mutant cells was due to increased viral replication, viral growth curves and quantitative PCR assays were performed. Cells were irradiated with a single 5 Gy fraction and 6 h later were infected with GLV-1h68 at an MOI of 1.0 (for measuring viral growth curves) or 0.01–1.0 (for A21L expression), and viral

replication was assessed at various time points for the viral growth curve and 48 h post-infection for A21L expression. The data from the viral growth curves as determined by TCID₅₀ or total viral titers

quantified by plaque assays on confluent CV-1 cells (Figures 3a and b respectively) and viral A21L expression measured by PCR (Figure 3c) clearly showed that the enhanced cell kill was not due

to an increase in viral replication when the cells were irradiated. Surprisingly, there was a clear reduction in viral replication in $V600D/E$ BRAF mutant cell lines following irradiation, while no change was noted in the wild-type or RAS mutant cells.

Having shown that viral replication was not responsible for increased cell death in irradiated $V600D/E$ BRAF mutant cells, we used a human phosphokinase array to investigate what proteins might be involved in mediating the interaction between RT and GLV-1h68 in MeWo and A375 melanoma cell lines (Figure 4a). Of note, there was an increase in p38, JNK and ERK phosphorylation in both the A375 ($V600E$ BRAF mutant) cell lines following GLV-1h68 infection. However, following RT, the phosphorylation of p38, JNK and ERK was abrogated in the A375 $V600E$ BRAF mutant cell line. In the MeWo cells, there was a substantial increase in JNK phosphorylation following GLV-1h68 infection, but little or no change in p38 or ERK phosphorylation, with RT having no effect on GLV-1h68-induced JNK phosphorylation (Figure 4a). We then confirmed the results of the kinase array of both MeWo and A375 cells using western blotting as shown in Figure 4b. Our western analysis in the A375 cells was consistent with the data from the kinase array where we saw abrogation of GLV-1h68-induced phosphorylation of p38, JNK and ERK following RT. The western analysis for the MeWo cells were slightly different from that seen in the kinase array, as GLV-1h68 showed a clear increase in p38 and ERK phosphorylation. Based on the consistent signal on the kinase array and western blot, we chose to focus on the effect of GLV-1h68 and RT on the JNK pathway.

Inhibition of the JNK pathway following irradiation results in increased cell death

To assess the role of JNK in regulating GLV-1h68 and RT-induced cell death, we carried out western blotting analysis (Figure 4c) to assess caspase 3 cleavage, a marker of apoptosis. We found modest or no increase in caspase 3 cleavage following GLV-1h68 infection in WM266.4 $V600D$ BRAF, A375 $V600E$ BRAF mutant cell lines. When GLV-1h68 was combined with RT, there was a marked increase in caspase 3 cleavage compared with GLV-1h68 or RT alone. When JNK phosphorylation status was assessed, we found that RT abrogated GLV-1h68-induced JNK phosphorylation in the $V600D/E$ BRAF mutant cell line. In addition, the other two $V600E$ BRAF mutant cell lines, Mel624 and SKMel28 showed a similar pattern where there was a marked increase in caspase 3 cleavage when GLV-1h68 was combined with RT. This correlated with a loss of GLV-1h68-induced JNK phosphorylation (Supplementary Figure S4B). Meanwhile, an increase in caspase 3 cleavage was seen following GLV-1h68 infection in wild-type (PMWK and MeWo), and N- and K-RAS mutant (D04 and WM1791, respectively) cell lines (Figure 4c). This was associated with an increase in JNK phosphorylation. In these cells, no increase in caspase 3 cleavage was seen following GLV-1h68 and RT treatment, while JNK phosphorylation remained unchanged.

We next determined whether the JNK pathway had a role in the mechanism of GLV-1h68- and RT-induced cell death in $V600D/E$ BRAF mutant cells; we used a specific JNK inhibitor (SP600125) in an attempt to mimic the effect of RT on GLV-1h68-induced cytotoxicity. Western analysis showed an increase in caspase 3

cleavage in A375 cells following RT and GLV-1h68 infection, as previously shown (Figure 5a, upper panel). Caspase 3 cleavage was also increased following GLV-1h68 infection combined with JNK inhibition, therefore, suggesting that JNK abrogation had a role in enhancing GLV-1h68- and RT-induced cell death. Similar results were obtained for three other BRAF mutants: WM266.4, Mel624 and SKMel28 cell lines (Figure 6a). In addition, two other RAS mutant cell lines; D04 and WM1791 and wild-type MeWo cells were tested and showed no effect (Figures 5a and 6a). Furthermore, JNK gene silencing by siRNA mimicked the results observed from the SP600125 inhibitor showing enhanced caspase cleavage when GLV-1h68 was combined with JNK siRNA in A375 cells, but not in MeWo or D04 cells (Figure 5a, lower panel).

Subsequently, by using SRB assay, we found that a combination of SP600125 and GLV-1h68 resulted in increased cell death compared with either agent alone in the A375, WM266.4, Mel624 and SKMel28 $V600D/E$ BRAF mutant cell lines (Figures 5b and 6b), with similar cell kill observed for GLV-1h68 combined with either SP600125 or RT. However, there was no enhanced cell death in wild-type MeWo cell lines or N- and K-RAS D04 and WM1791 cell lines, respectively, when SP600125 was combined with GLV-1h68—in keeping with the data for GLV-1h68 combined with RT (Figures 5b and 6b). Crystal violet staining was also carried out and confirmed the response patterns described in the SRB assay (Supplementary Figure S5). Additionally, using the SRB assay, we found that gene silencing of JNK in combination with GLV-1h68 enhanced cell death compared with either agent alone in A375 $V600E$ BRAF mutant cell line (Figure 5b, lower panel). However, there was no increase in cell death in either wild-type MeWo or D04 N-RAS mutant cell lines following JNK silencing and GLV-1h68 infection. As was the case for GLV-1h68 in combination with RT, GLV-1h68 in combination with SP600125 attenuated viral replication in $V600D/E$ BRAF mutant, but not in wild-type or RAS mutant cell lines (Figures 5c, d and 6c).

RT or JNK inhibition reduces TNF- α secretion following GLV-1h68 infection in $V600D/E$ BRAF mutant melanoma cells only

TNF- α is a pleiotropic cytokine that has a central role in inflammation and apoptosis.¹⁹ Paradoxically, it has previously been shown to act as a survival signal in $V600D$ BRAF and $V600E$ BRAF mutant melanoma cell lines, where it prevented MEK inhibitor-induced cell death.²⁰ Using a human TNF- α ELISA, we were able to show that TNF- α secretion was increased after GLV-1h68 infection in both $V600D/E$ BRAF mutant and wild-type melanoma cell lines. Following RT, TNF- α secretion was reduced in A375 $V600E$ BRAF and WM266.4 $V600D$ BRAF mutant cell lines, but no reduction was seen in wild-type cells (Figure 7a). To test if the abrogation of GLV-1h68-induced TNF- α secretion in $V600E$ BRAF mutant cell lines was JNK-mediated, we used the specific JNK inhibitor, SP600125 and TNF- α monoclonal antibody. We found that GLV-1h68-induced TNF- α secretion was abrogated by SP600125 in A375 $V600E$ BRAF mutant, but not wild-type MeWo cells (Figure 7b). These findings mirror the effects seen with RT and SP600125. Using western blotting analysis to detect caspase 3 cleavage, crystal violet staining and SRB assay to measure cell death, we found that a combination of TNF- α monoclonal antibody and GLV-1h68 resulted in increased

Figure 1. Combined GLV-1h68 and RT treatment in a panel of melanoma cells lines. Cells were irradiated at 1, 3 and 5 Gy and 6 h later infected with GLV-1h68 at different MOIs. Cell survival was measured using MTT assays at 72 h post-infection (a) and synergy assessed using the Bliss Analysis method (b). Cell survival was further validated using the SRB assay (c) at 72 h post-infection. Results are shown from three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. Clonogenic assays were carried out to assess RT effect in MeWo, A375 and D04 cell lines. MeWo, A375 and D04 cells were plated at an appropriate seeding density and allowed to attach. Twenty-four hours later, cells were irradiated at 2 and 5 Gy. Colony formation was determined at 10–14 days irradiation and the surviving fractions calculated relative to the control. Data are shown from two independent experiments (d). Cells were plated at 5×10^5 in a T25 flask and irradiated at 2 Gy after 24 h. GLV-1h68 was then infected at an MOI of 0.03. Forty-eight hours later, cells were washed in PBS, trypsinized and counted. An equal number of cells were plated from each treatment group into six-well plates. Colony assays were stained with crystal violet, 10–14 days later(e).

cell death compared with either agent alone in the A375^{V600E} BRAF mutant cell lines, compared with wild-type MeWo cells (Figures 7c, d and e, respectively).

DISCUSSION

Metastatic malignant melanoma (MMM) is a highly aggressive tumor that is largely refractory to conventional anticancer

chemotherapy.^{21–24} It is classically regarded as relatively resistant to RT therapy.¹³ As a result, for many years, most oncologists have viewed MMM nihilistically as an essentially untreatable disease. However, recent studies have shown that MMM can be divided into specific molecular pathological subtypes that may be amenable to specific targeted therapies. For example, the finding that ~50% of melanomas harbor oncogenic BRAF mutations has driven drug discovery programmes that have generated potent, selective inhibitors that are active against V600E BRAF mutant tumor cells.^{25–27} These agents have yielded dramatic results in clinical trials in patients with V600E BRAF mutant melanoma.^{28,29} The BRIM-3 phase III study recently reported response rates of 48% for PLX4032 (vemurafenib) versus 5% for the standard-of-care (dacarbazine) in patients with V600E BRAF mutant melanoma.¹⁴ These data translated to a 20% advantage for vemurafenib in terms of overall survival at 6 months. However, therapeutic responses were usually short-lived (<1 year) and frequently associated with aggressive disease recurrence. These observations highlight the need for continued research effort into novel therapies for BRAF mutant melanoma. As a note of caution, however, the importance of a detailed understanding of the molecular pathology of MMM was underlined in preclinical work that showed that, in BRAF wild-type tumor cells, the use of a BRAF inhibitor can activate signaling through BRAF-CRAF dimerization and, as a result, may accelerate tumor growth.²⁷ Therefore, future development of specific targeted therapies for MMM must include a detailed analysis of the various genetic backgrounds of this disease.

In the context of this evolving understanding of the molecular pathology of MMM, we have assessed the combined effect of GLV-1h68 and RT in a panel of melanoma cell lines of varying genetic backgrounds (V600D BRAF mutant, V600E BRAF mutant, K-RAS mutant, N-RAS mutant and wild-type for BRAF, K-RAS and N-RAS). We hypothesized that constitutive activation of JNK signaling would render RAS or BRAF mutant melanoma cell lines highly susceptible to vaccinia viral replication and oncolysis. Furthermore, we posited that RT-induced activation of JNK signaling would further enhance replicative viral cytotoxicity in all genetic backgrounds.

Contrary to expectations, BRAF/RAS wild-type melanoma cell lines were more sensitive to GLV-1h68 than the BRAF or RAS mutants (Supplementary Figure 1A) and only the 4 V600D/E BRAF mutant cell lines showed enhanced cell death when GLV-1h68 was combined with RT *in vitro* (Figures 1a–d and Supplementary Figure S2) and *in vivo* (Figure 2). These findings suggested a complex combinatorial interaction between vaccinia virus and RT in melanoma cell lines that is governed by the underlying molecular pathology of the disease. Therefore, in subsequent experiments, we sought to uncover the mechanisms underpinning the differential effects of vaccinia virus and RT in V600D/E BRAF mutant, compared with RAS mutant or BRAF/RAS wild-type, melanoma cells.

In line with the received wisdom that oncolytic viruses kill by viral replication, we hypothesized that the increased cell death in V600D/E BRAF mutant melanoma cells treated with vaccinia virus

and RT would be explicable in terms of enhanced viral replication. Certainly, a number of previous studies with different viruses have reported increased viral replication linked to enhanced lytic cytotoxicity following combination treatment with either RT or cytotoxic chemotherapy.^{30–32} However, others have reported no change or a reduction in viral replication in combination regimens with RT or chemotherapy.^{23,33–37} Our data clearly demonstrate by both viral plaque assay and quantitative PCR that the enhanced cytotoxicity of the combined treatment in V600D/E BRAF mutant cells was not due to increased viral replication. Paradoxically, the increased levels of cell death were associated with a definite decrease in viral replication in V600D/E BRAF mutant cells (Figure 3). In contrast, there was no difference in viral replication in RAS mutant or wild-type melanoma cell lines.

Having established that the synergistic effect of the combined treatment in V600D/E BRAF mutant cells was not due to increased viral replication, we examined the activity of intracellular signaling pathways in response to viral infection, irradiation and the combination of infection and irradiation. Vaccinia virus is known to manipulate host-signaling pathways, such as MAPK³ and Akt^{38,39} pathways, to enhance viral replication. Using a kinase array, we showed that infection with GLV-1h68 activated JNK pathways in both V600E BRAF mutant and BRAF/RAS wild-type cells. Critically, following irradiation, JNK signaling was abrogated in V600E BRAF mutant A375 cells, but unaltered or further increased in wild-type MeWo cells (Figure 4a). This observation may, in part, explain the reduced viral replication seen after irradiation in V600D/E BRAF mutant cells. Our data suggest that the abrogation of p-JNK signaling in V600D/E BRAF mutant cells is associated with reduced vaccinia virus replication but increased levels of apoptosis, with the net effect being synergistic cytotoxicity.

We subsequently demonstrated that pharmacological inhibition of JNK phosphorylation recapitulated the effects of RT in terms of cytotoxicity (Figures 5b and 6b), viral replication (Figures 5c, d and 6c) and caspase cleavage (Figures 5a and 6a). These findings suggest that RT-induced inhibition of JNK phosphorylation in V600D/E BRAF mutant melanoma cells has a key role in mediating the differential effects of RT in the different genetic backgrounds of melanoma.

Having seen differential signaling in JNK cascades in V600D/E BRAF mutant and wild-type cells following the combination of vaccinia virus and RT, we focussed on understanding potential causes and effects of these changes. Previous studies have shown a role for TNF- α signaling in the tumor microenvironment in *de novo* skin carcinogenesis⁴⁰, and TNF- α has been shown to activate MAPK (p38MAPK, ERK and JNK) pathways.^{41,42} Therefore, we investigated TNF- α signaling following GLV-1h68 infection alone and in combination with RT. These studies revealed that melanoma cells harboring a V600D/E BRAF mutation were relatively resistant to GLV-1h68-induced cell death and that this was associated with TNF- α secretion and activation of p38MAPK, ERK and JNK. However, following irradiation, GLV-1h68-induced TNF- α secretion was significantly reduced in V600E BRAF mutant, but not wild-type, cells with coordinate reduction in p38MAPK, ERK and JNK signaling and enhanced cytotoxicity. These data are in line with previous work

Figure 2. Combined GLV-1h68 and RT enhances tumor reduction and animal survival in V600E BRAF mutant melanoma *in vivo*. A375 subcutaneous xenograft tumor models were established in nude mice. (a) Mice were either untreated (PBS), irradiated at 2 Gy/fraction for three doses, GLV-1h68-infected at 1×10^4 PFU or combined treatment (2 Gy/fraction for three doses + 1×10^4 PFU GLV-1h68). (b) Size of the tumors were measured for each treatment group. Each bar represents mean $\pm$ s.e. of 10 replicates. (c) Comparison of mean tumor volumes between treatment groups at serial time points after and including day 45 using a Mann–Whitney *U*-test. There was significant inhibition of tumor growth in the GLV-1h68 and RT combination groups compared with other groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. (d) Kaplan–Meier curve was evaluated for each treatment group to assess the median survival rate. There was significant prolongation of survival in the combination of RT and GLV-1h68 compared with either agent alone. ** $P = 0.0099$. (e) Immunoblot of caspase 3 cleavage, a biomarker of apoptosis was carried out following homogenization of A375 xenografts, 7 days post-treatment from each treatment arm. Three mice from each treatment group were used in this experiment. Equal loading of proteins was assessed by probing for GAPDH. (f) Tumor-frozen sections were used for TUNEL staining immunohistochemistry analysis.

Figure 3. RT does not increase GLV-1h68 viral replication. Cells were irradiated at 5 Gy and 6 h later infected with GLV-1h68. The cells were harvested and the supernatants were collected at 4, 24, 48 and 72 h post-infection in triplicate. Viral titers were determined by using one-step growth curves (**a**). Cells were irradiated at 5 Gy and 6 h later infected with GLV-1h68 at an MOI of 1.0. Standard viral plaque assays were performed to assess viral replication in melanoma cells. Supernatants were collected 48 h later and total viral titers were quantified by plaque assays on confluent CV-1 cells (**b**). Cells were also analyzed for the presence of GLV-1h68 using vaccinia virus A21L-specific primers (**c**).

from other laboratories that have shown that TNF- α can function as a survival signal in V^{600D}/E BRAF mutant melanoma cells lines.¹⁷ Therefore, we hypothesize that inhibition of GLV-1h68-induced

TNF- α and JNK pathways by RT or pharmacological (SP600125) means removes essential survival signals and results in an increase in V^{600D}/E BRAF mutant cell death (Figure 7d).

Figure 4. Enhanced cell death following RT and GLV-1h68 is regulated by the JNK signaling pathways. Phosphokinase array in MeWo and A375 cells to assess how the MAPK family kinases are regulated following RT and GLV-1h68 treatment. MeWo (upper left panel) or A375 (upper right panel) cells were either untreated, GLV-1h68-infected (MOI of 0.1), irradiated (5 Gy) or combined (5 Gy + GLV-1h68 at an MOI of 0.1) and harvested after 72 h. Cell lysates were prepared and 0.2 mg of total protein was applied to phosphokinase arrays, which expressed the MAPK signaling pathway (a). Western blot analysis of phospho-JNK, phospho-p38, and phospho-ERK in untreated, GLV-1h68, RT or RT + GLV-1h68 were validated in MeWo and A375 cells to confirm results from kinase array. Equal loading of proteins was assessed by probing for GAPDH (b). Western analysis to assess JNK activation and cleaved caspase-3, a marker of apoptosis in melanoma cells following irradiation at 5 Gy and GLV-1h68 infection between an MOI of 0–0.25 (MeWo, PMWK, A375 and WM266.4) and MOI of 0.1 (WM1791 and D04) cells. Equal loading of proteins was assessed by probing for GAPDH (c).

In summary, our preclinical studies have provided a strong rationale for clinical translation of GLV-1h68 in combination with irradiation in $V^{600D/E}$ BRAF mutant cells, while also supporting the use of GLV-1h68 monotherapy in RAS mutant and BRAF/RAS wild-type melanoma. We are currently attempting to dissect how and why RT regulates vaccinia-induced TNF- α /JNK survival pathways in $V^{600D/E}$ BRAF mutant, but not wild-type or Ras mutant cells, and how this mediates enhanced cell death. In addition, we are further characterizing other signaling alterations that were identified following GLV-1h68 and RT treatment, in particular, the p38 MAPK and ERK pathways.

MATERIALS AND METHODS

Cell lines

Melanoma cell lines of known genetic background were obtained from Professor Richard Marais (The Paterson Institute of Cancer Research). The following cell lines were used: A375, Mel624, SKMel28 (all V^{600E} BRAF mutant), WM266.4 (V^{600D} BRAF mutant),

Mewo and PMWK (wild-type RAS and BRAF), D04 (N-RAS mutant) and WM1791 (K-RAS mutant). All the cell lines were authenticated by using short tandem repeat profiling carried out by Bio-Synthesis Inc (Lewisville, TX, USA) within the last 6 months. For simplicity, throughout the rest of this manuscript, BRAF mutant cell lines, which are either V^{600D} BRAF or V^{600E} BRAF mutant will be referred to as $V^{600D/E}$ BRAF mutant. Cells were cultured in DMEM. Media was supplemented with 5% (v/v) FCS, 1% (v/v) glutamine and 0.5% (v/v) penicillin/streptomycin.

Oncolytic vaccinia (GLV-1h68)

GLV-1h68 is a genetically engineered Lister strain vaccinia that was generated by inserting three expression cassettes (encoding *Renilla* luciferase-*Aequorea* green fluorescent protein (GFP) fusion protein, β -galactosidase (β -gal), and β -glucuronidase) into the *F14.5L*, *J2R*, and *A56R* loci of the LVP (Lister strain from the Institute of Viral Preparations, Moscow, Russia) strain viral genome, respectively.

Figure 5. JNK mediates GLV-1h68- and RT-enhanced apoptosis in $V_{600D/E}$ BRAF mutant melanoma. MeWo (wild-type), A375 (V_{600E} BRAF mutant) and D04 (N-RAS mutant) cells were incubated with 1 μ M of JNK inhibitor SP00125 for 4 h and radiated at 5 Gy and then infected with GLV-1h68 after 6 h at an MOI of 0.1 (upper panel), or transfected with 5 and 10 nM JNK siRNA or scramble control for 4 h and then infected with GLV-1h68 at an MOI of 0.1 (lower panel). Cell lysates were assessed for cleaved caspase 3 and phospho-JNK at 72 h by immunoblotting. Equal loading of proteins was assessed by probing for GAPDH (a). Cell viability was carried out using SRB assay (b). Results are shown from three independent experiments. *** $P < 0.005$. Viral plaques from MeWo and A375 cell lines were stained by β -galactosidase and visualized microscopically (c), while viral replication was validated using one-step growth curve assays (d).

Figure 6. A panel of melanoma cells were incubated with 1 μ M of JNK inhibitor SP00125 for 4 h and radiated at 5 Gy. Cells were then infected with GLV-1h68 after 6 h at an MOI of 0.1 and lysates probed for cleaved caspase 3 and phospho-JNK at 72 h by immunoblotting. Equal loading of proteins was assessed by probing for GAPDH (**a**). The effect of JNK inhibition and RT on cell viability and viral replication were assessed in WM266.4, Mel624, SKMel28 (^{V600D}/BRAF mutant) and WM1791 (K-RAS mutant) cells using SRB assay (**b**) and one-step growth curve assays (**c**).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
Cell viability was quantified using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 20 μ l MTT (thiazolyl blue; Sigma-Aldrich) at 5 mg/ml in PBS was added to treated cells in a 96-well plate. After 4 h incubation at 37 °C, crystals were solubilised in DMSO and absorbance was measured at 570 nm on a SpectraMax 384 plate reader (Molecular Devices, Berkshire, UK).

Crystal violet and sulforhodamine B assays

Cell viability was quantified by staining either with crystal violet (Sigma-Aldrich, Dorset, UK) at 0.2% (w/v) in a 7% (v/v) solution of ethanol/PBS or 10% trichloroacetic acid (TCA) and stained with sulforhodamine B (SRB). The crystal violet-stained images of the plate were captured on a Microtek ScanMaker 8700 (Microtek International Ltd, Hsinchu, Taiwan), while the SRB stained cells were diluted with 1 mM TRIS and absorbance was measured at 570 nm on a SpectraMax 384 plate reader.

Clonogenic assays

For studies to determine the effect of RT at 0, 2 and 5 Gy, melanoma cells were plated on a six-well plate (density was dependent on plating efficiency), and 24 h later, RT was delivered. After 10 to 14 days, colonies were fixed, stained and counted. Cell survival was determined by normalization to the untreated control after correcting for the plating efficiency. For studies involving GLV-1h68 in combination with RT, cells were plated at 5×10^5 in a T25 flask. The next day, cells were irradiated at 2 Gy and 6 h later

infected with GLV-1h68 at an MOI of 0.03. Forty-eight hours post-treatment, cells were washed in PBS, trypsinized and counted using a haemocytometer. Cells were then plated into six-well dishes at 400–800 cells/well. After 10–14 days, plates were stained with 0.2% crystal violet in 7% ethanol, and the plates containing the colonies were scanned.

One-step growth curve assays

Titers of GLV-1h68 in infected melanoma cells (with or without RT) were determined. Briefly, melanoma cells were seeded in 24-well plates at a density of 2×10^5 cells/well and infected with GLV-1h68 at an MOI of 1. The cells were harvested and the supernatants were collected at 4, 24, 48 and 72 h post-infection, in triplicate. After three freeze–thaw cycles between –80 °C and room temperature, the resulting lysates or viral suspensions were diluted in 10-fold series and used to infect CV-1 cells. Viral titers were determined by limited dilution using the TCID₅₀ method.

Viral plaque assays to determine viral titers

Melanoma cells were plated at a density of 2×10^5 cells/well in 24-well plates. Culture plates were irradiated the next day at 5 Gy and infected with GLV-1h68 at an MOI of 1. Supernatant from each well was collected at 48 h post-infection and the viral plaques from the infected cells released by three freeze–thaw cycles. CV-1 cells were grown to confluence on 24-well plates. Supernatants were thawed, and serial dilutions incubated on the CV-1 cells for 4 h. Wells were washed with media and incubated with fresh media. After 48 h of incubation, virus detection was carried out on

Figure 7. GLV-1h68 treatment results in increased TNF- α secretion and is abrogated following irradiation in a $V600D/E$ BRAF mutant melanoma mutant population. **(a)** MeWo, PMWK, A375 and WM266.4 cells were irradiated at 5 Gy and then infected with increasing doses of GLV-1h68. Forty-eight hours later, TNF- α that was secreted into the medium was measured using a TNF- α ELISA. Results are shown from three independent experiments. * $P < 0.05$; *** $P < 0.005$. **(b)** MeWo and A375 cells were incubated with 1 μ M of JNK inhibitor SP00125 or 2 ng per ml of TNF- α monoclonal antibody for 4 h and irradiated at 5 Gy. Cells were then infected with GLV-1h68 after 6 h at an MOI of 1 (for TNF- α ELISA) or 0.1 (viral plaque assay). TNF- α that was secreted into the medium was measured using a TNF- α ELISA following 72 h of infection (left panel). Results are shown from three independent experiments. *** $P < 0.005$. **(c)** Caspase 3 cleavage and JNK activation were measured by western blotting analysis. Equal loading of proteins was assessed by probing for α -tubulin **(d)**. Cell viability was measured using crystal violet assay **(d)** and SRB assay **(e)**. A model of cytotoxicity in $V600D/E$ BRAF mutant melanoma cells mediated by JNK following irradiation and GLV-1h68 treatment **(f)**.

the cell monolayers. Briefly, cells were washed with PBS, fixed with 0.2% Glutaraldehyde/2% formaldehyde for 5 min, and thereafter washed with PBS. Cells were stained for 4 h in X-galactosidase (0.6 mg/ml) in a solution of 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ and 2 mM $MgCl_2$, and then, the viral plaques were counted and the viral titers determined. In addition, viral plaques from MeWo and A375 cell lines were also captured on a Microtek ScanMaker 8700 or examined by microscopy ($\times 20$).

A21L expression

qPCR using the Genelux GL-LC1 VV-A21L kit (Genelux GmbH) was used for semiquantitative detection of the vaccinia A21L gene. DNA was prepared from the media of melanoma cells following infection with GLV-1h68 $\pm$ irradiation at 48 h. A21L-specific primers were used (forward: 5'-CGTAACTACAAACGTCTAAACAA GAA-3' and reverse: 5'-CCTGGTATATCGTCTCTATCTTTATCAC-3'). The 18S rRNA of human/rat genomic DNA (18S) was used as a reference.

Kinase array

The activity of 46 kinase phosphorylation sites was assessed using an antibody-based array from R&D Systems (Abingdon, UK). Cell lysates were obtained from A375 or MeWo cells exposed to the following experimental conditions: untreated (no virus, no RT); virus only (GLV-1h68 virus infected, no RT); RT only (no virus, irradiated to 5 Gy); and combined virus and RT (infected with GLV-1h68 and irradiated to 5 Gy).

Small interfering RNA transfections

Scramble control and JNK1/2 siRNA small interfering RNAs (siRNA) were obtained from Thermo Fisher Scientific, Waltham, MA, USA. MeWo, A375 and D04 cells were seeded out in the appropriate media without penicillin-streptomycin. Twenty-four hours after seeding, siRNA transfections were done on subconfluent cells incubated in unsupplemented OptiMEM using the Lipofectamine reagent (Invitrogen, Paisley, Scotland, UK) according to the manufacturer's instructions. After 4 h, $3 \times$ growth medium was added to the media. Cells were then infected with GLV-1h68 (MOI of 0.1) for 72 h and the lysates collected for western analysis or assessed for cell death by SRB assay.

Western blotting

Cells were plated at 0.5×10^6 in 60-mm dishes. Following various treatments, cells were harvested in ice-cold PBS, pelleted and resuspended in radioimmunoprecipitation assay buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS) with protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany), 1 mM sodium orthovanadate (Sigma), and 10 mM sodium fluoride. Cells were then lysed by snap freezing on dry ice and then allowing the lysate to thaw on ice for 10 min. The lysate was then centrifuged at 13 200 r.p.m./4 °C for 20 min to remove cell debris. Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL, USA). Thirty micrograms of each protein sample were resolved on SDS-polyacrylamide gels (10–12%) and transferred to a polyvinylidene difluoride Hybond-P membrane (Amersham, Buckinghamshire, UK). Immunodetections were performed using anti-phosphorylated JNK (Thr 183/Tyr 185, Cell Signaling, Beverly, MA, USA), anti-phosphorylated p38 MAPK (Thr 180/Tyr 182, Cell Signaling), anti-phosphorylated p44/42 ERK MAPK (Thr 202/Tyr 204, Cell Signaling) and caspase 3 (Cell Signaling) rabbit polyclonal antibody in conjunction with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (GE-Healthcare, Buckinghamshire, UK). Equal loading was assessed using α -tubulin (Sigma-Aldrich) or glyceraldehyde-3-phosphate dehydrogenase-GAPDH (Biogenesis) mouse monoclonal primary antibodies. The Super Signal chemiluminescent system (Pierce) or Immobilon western

chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) were used for detection.

In vivo studies

A375 tumors were established in female CD1 nude mice by subcutaneous injection of 5×10^6 cells suspended in 100 μ l PBS in the right flank. Once xenografts were established and reached ~ 5 mm in diameter, mice were randomly allocated to treatment groups (14 mice per group) before beginning therapy. Irradiation of mice was carried out as previously described.³² Briefly, mice received an intraperitoneal injection of 100 μ l of a 1:1:4 mixture of Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone; Janssen-Cilag Ltd., Suffolk, UK), Hypnovel (5 mg/ml midazolam; Roche Products Ltd.), and water for injection BP (Fresenius Health Care Group, Kent, UK) prior to irradiation. Control animals were also anesthetized in the same way. Anesthetized animals were positioned in an irradiation jig with the subcutaneous tumors exposed under an aperture in a 3-mm lead sheet that shielded the rest of the body. Following irradiation, to limit hypothermia to a minimum, animals were wrapped in toweling jackets until they recovered consciousness (~ 30 –60 min). Local fractionated RT treatment of the tumor consisted of a total dose of 6 Gy in three fractions over 5 days (Figure 2). GLV-1h68 was administered by intratumoral injection 1 h before the second fraction of RT dose. Four mice from each group were killed after 7 days treatment and tumors harvested for Caspase 3 cleavage (three mice/group) and TUNEL staining (one mouse/group). Tumors from the remaining mice (10 mice per group) were measured twice weekly in two dimensions using Vernier callipers and the volume estimated using the formula (width² $\times$ length)/2. As per our institutions, animal licensing regulations humane end point was defined as a tumor diameter > 15 mm in any dimension.

TUNEL assay and caspase 3 cleavage

For *in vivo* analysis of apoptosis, tumors were harvested from mice 7 days after treatment and analyzed for TUNEL staining (one mouse per group) and by western blot analysis for activated caspase 3 (three mice per group). Tumors harvested for immunohistochemistry staining were fixed in 10% formalin for 24 h and then transferred to PBS and stored at 4 °C until analyzed. Tumors for western analysis were snap-frozen and homogenized for two cycles of 10 s in RIPA buffer. Samples were then centrifuged at 13 000 r.p.m. for 20 min at 4 °C and the supernatant transferred to a fresh tube. Western analysis was carried out as described previously (in 'Western blotting' methods above).

Statistical analysis

Comparisons between groups were done using the two-way analysis of variance (*in vitro* analysis) or a Mann–Whitney *U*-test (*in vivo* analysis). Survival curves were estimated using the Kaplan–Meier method, and significance was assessed using the log-rank test. *P*-values < 0.05 were considered to be statistically significant (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$). Synergy interactions between different treatments (for example, GLV-1h68 and RT) were tested by standard mathematical analyses of data from MTT assays. Specifically, the presence (or absence) of synergy was quantified by Bliss Independence Analysis^{15–18} described by the formulae $E_{IND} = E_A + E_B - E_A \times E_B$ and $\Delta E = E_{OBS} - E_{IND}$ where: E_A and E_B are the fractional effects of factors A and B, respectively; E_{IND} is the expected effect of an independent combination of factors; E_{OBS} is the observed effect of the combination. If ΔE and its 95% CI were > 0 , synergy was observed. If ΔE and its 95% CI were < 0 , antagonism was observed. If ΔE and its 95% CI were 0, then the combination was independent. All plots were generated using Prism GraphPad software (La Jolla, CA, USA).

CONFLICT OF INTEREST

Aladar Szalay is president and a shareholder of GeneLux corporation. Kevin Harrington receives funding from GeneLux corporation in support of laboratory research. The remaining authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (<http://www.nature.com/onc>)