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Oncolytic herpes simplex virus vectors and chemotherapy: are combinatorial strategies more effective for cancer?

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Abstract

Despite aggressive treatments, including chemotherapy and radiotherapy, cancers often recur owing to resistance to conventional therapies. Oncolytic viruses such as oncolytic herpes simplex virus (oHSV) represent an exciting biological approach to cancer therapy. A range of viral mutations has been engineered into HSV to engender oncolytic activity. While oHSV as a single agent has been tested in a number of cancer clinical trials, preclinical studies have demonstrated enhanced efficacy when it is combined with cytotoxic anticancer drugs. Among the strategies that will be discussed in this article are combinations with standard-of-care chemotherapeutics, expression of prodrug-activating enzymes to enhance chemotherapy and small-molecule inhibitors. The combination of oHSV and chemotherapy can achieve much more efficient cancer cell killing than either single agent alone, often through synergistic interactions. This can be clinically important not just for improving efficacy but also for permitting lower and less toxic chemotherapeutic doses. The viral mutations in an oHSV vector often determine the favorability of its interactions with chemotherapy, just as different cancer cells, due to genetic alterations, vary in their response to chemotherapy. As chemotherapeutics are often the standard of care, combining them with an investigational new drug, such as oHSV, is clinically easier than combining multiple novel agents. As has become clear for most cancer therapies, multimodal treatments are usually more effective. In this article, we will discuss the recent progress of these combinatorial strategies between virotherapy and chemotherapy and future directions.

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Oncolytic viruses (OVs) are replication-competent viruses that have been selected or engineered to replicate in tumor cells but not in normal cells [1]. Principally, OVs destroy tumor cells as a result of lytic infection, while sparing normal tissues. Virotherapy, the use of viruses as oncolytic agents for cancer therapy, is an old concept that has been tested in humans since the 1950s [2]; however, progress was only recently possible with advances in molecular biology, genetics and virology allowing for the genetic engineering of OVs or the identification of naturally occurring viruses with intrinsic tumor selectivity [3]. The first genetically-engineered OV was an oncolytic herpes simplex virus (oHSV) [4]. Since the first clinical trials with oncolytic adenovirus ONYX-015® (Onyx Pharmaceuticals Inc., CA, USA) in 1998 [5], OVs from at least seven different virus species – adenovirus, HSV, vaccinia virus, reovirus, Newcastle disease virus, Seneca Valley virus and measles virus – have been assessed in clinical trials [6].

Oncolytic HSV virotherapy: recent progress & limitations

Herpes simplex virus is a natural pathogen to humans, and infection can cause lethal encephalitis. It is an enveloped virus, approximately 200 nm in diameter, with a dsDNA genome of approximately 153 kbp encoding approximately 90 genes [7]. The virus attaches to the cell surface through heparan sulfate proteoglycans, followed by envelope glycoprotein interactions predominantly with receptors HveA and nectin-1, and also by membrane fusion. Capsids are then transported to the nucleus, where the DNA is released and initiates a lytic replication cycle. There is a stereotypic temporal cascade of gene expression; first, immediate early proteins, which drive transcription and protein synthesis of early genes that are required for DNA replication, and second, after DNA replication, a switch to late gene expression, encoding mostly structural proteins [7]. In some neurons, the virus can undergo a latent infection, with minimal gene expression and no cytotoxicity. oHSV has a number of advantages over OVs based on other viruses, including:

- It infects most cell types in a broad range of species;
- It is very cytolytic, so that one infectious particle can replicate and spread in a cell monolayer;
- Its genome is very stable;
- Many nonessential genes contribute to pathogenicity and replication in nondividing cells;
- It has a large genome, allowing multiple, non-essential genes to be replaced (up to 30 kbp) with multiple therapeutic transgenes;
- Effective antiviral drugs are available to treat adverse events in patients [8,9].

No less than six different oHSVs have already undergone or will be entered into clinical trials worldwide for a variety of different cancers, with some having progressed to Phase II/III trials (Table 1). Early development of oHSVs focused on the construction of ‘safe’ vectors. This included the deletion of the viral *γ34.5* gene (e.g., R3616 and 1716), the major neuropathogenicity gene in HSV, to minimize neurotoxicity. This was followed by the introduction of multiple deletions and/or mutations to prevent reversion to wild-type virus (e.g., G207) [10]. As expected, these early-generation oHSVs (G207 and 1716) have demonstrated excellent safety profiles in clinical trials [11,12]. Clinical trials with G207 and 1716 for glioma

found evidence of virus replication, but only to a limited degree [11,13], suggesting that enhancement of oncolytic activity and virus replication would be beneficial for therapy.

To date, no serious adverse events solely attributable to oHSV have been reported in the clinical trials, including when administered by intra-cerebral injections for glioma treatment. Thus, the deletions or mutations confer significant safety and selectivity to tumor cells onto oHSV; however, efficacy has been attenuated. In addition to attenuation of oncolytic potency, multiple factors may contribute to the suboptimal efficacy of oHSV *in vivo*. Direct intratumoral injection of oHSVs has been the preferred route of administration, and this limits delivery to accessible tumor sites. Physical barriers such as the extracellular matrix can restrict initial oHSV distribution and subsequent spread of virus in the tumor mass, allowing tumor cells to 'outgrow' the virus [14]. Both innate and acquired anti-HSV immunity can clear oHSV prematurely to also limit oHSV replication and spread [15,16]. During the last decade, there has been steady progress in the development and translation of new oHSVs into the clinic. The recent success of OncoVEX$^{GM\text{-}CSF}$ in a Phase II clinical trial for melanoma has provided the impetus to proceed to a pivotal Phase III randomized trial, the first for an oHSV [17]. An important component of this therapy is immune mediated, and most tumors are not as responsive to immunotherapy as melanoma; thus, additional strategies to enhance efficacy are still needed. These include generation of oHSVs with increased potency, construction of oHSVs carrying therapeutic transgenes (e.g., immune-stimulatory molecules and prodrug-activating enzymes) and oHSV combinations with chemotherapy or radiotherapy. This article will focus on oHSV combinations with chemotherapy.

Virotherapy–chemotherapy combinations

The therapeutic index for chemotherapeutic drugs is rather narrow, and this, coupled with severe dose-limiting toxicities and emergence of resistance, restricts their effectiveness. This is in contrast to OVs, to which cancer cells do not seem to develop resistance and which have a large therapeutic index with limited toxicities. Thus emerges a rationale to combine these two modalities, with different mechanisms of action, in order to increase cancer cell killing, while minimizing toxic side effects to normal tissues (i.e., to widen the therapeutic window) [18]. In addition, there is the likelihood that OVs and chemotherapy will interact positively and/or synergistically, which would be highly beneficial to the patient [18–20]. The first report of augmenting OV activity with chemotherapy was in 1997 using adenovirus vector ONYX-015 and cisplatin (CDDP) or 5-fluorouracil (5-FU) [21]. The combination with CDDP and 5-FU was then translated to a Phase II clinical trial for head-and-neck cancer [22]. ONYX-015 has also been evaluated clinically in combination with; 5-FU and leucovorin [23], MAP chemotherapy (i.e., mitomycin C [MMC], doxorubicin [Adriamycin®; ADR] and CDDP) [24], irinotecan and 5-FU [25] and gemcitabine [26]. It was striking that ONYX-015 by itself did not show objective response; however, combinations with chemotherapy showed marked responses in some tumor types, even using chemotherapy that had previously failed in the patient [5]. In 2005, a similar oncolytic adenovirus construct, H101, was approved in China for use in treating nasopharyngeal carcinoma in combination with 5-FU and CDDP [27].

Since then, additional oncolytic adenovirus vectors [28–30] and a number of other OVs have been tested with conventional chemotherapies in preclinical studies, and some of these combinations have been translated to clinical trials [18]. Synergy between reovirus and CDDP or gemcitabine was demonstrated *in vitro* in four out of six human non-small-cell lung cancer (NSCLC) lines, dependent upon the drug sensitivity of the cells [31]. Even greater synergy was observed with paclitaxel. Oncolytics Biotech Inc. (AB, Canada) is currently conducting clinical trials with reovirus in combination with gemcitabine (NCT00998322) and carboplatin/paclitaxel (NCT00998192, NCT00984464, NCT00861627 and NCT00753038) [201].

The combination effect will depend upon: the type of cytotoxic damage mediated by the drug; the cellular response, usually a DNA-damage response and/or cell cycle arrest; the form of cell death (e.g., apoptosis, necrosis or autophagy); and drug- or virus-resistance mechanisms.

oHSV–drug combinations

There are a number of features of oHSV therapy that imply that combination with chemotherapeutic drugs would be beneficial:

- The mode of action of oHSV toxicity is distinct from conventional chemotherapies;
- The mode of action of oHSV toxicity is independent of many of the genomic alterations that are observed in chemotherapy-resistant tumors, such as in *p53* [8, 32];
- The cancer selectivity of oHSV should limit any increases in chemotherapy-mediated cytotoxicity to the tumor, thus improving the therapeutic index;
- Differing toxicological profiles suggest that the side effects will not overlap.

To date, there have been no reports of cancer-cell crossresistance to chemotherapy and oHSV. Importantly, in most cases, chemotherapy-resistant cancer cells demonstrate similar susceptibility to oHSV cytotoxicity as sensitive cells. For example: CDDP-resistant human head-and-neck squamous cell carcinoma (SCC) and ovarian carcinoma cells, and oHSV G207 and R3616, respectively [32,33]; 5-FU-resistant colon carcinoma cells and NV1020 [34]; temozolomide (TMZ)-resistant glioma cells and G207 [35]; and flutamide-resistant (androgen independent) human prostate tumors and G47Δ [36]. See Table 1 for a description of the genotype of the different oHSVs discussed in the text. As an insult to cells, each agent elicits alterations to intracellular signaling/metabolic pathways, which can merge and influence the overall outcome depending upon the specific drug or virus.

Experimental analysis of how two agents interact in terms of tumor cell killing *in vitro* has typically been determined using the median-effect method of Chou and Talalay [37] and isobologram or combination index equations [38–40]. The drug and virus are added to cells in combination ratios equaling the ratio of their median-effect doses, derived from individual dose–response curves. The combined dose–response curve is fitted to a Chou–Talalay line and combination indices determined. Synergy is usually defined as an effect that is more than additive, and combination index values of 0.9–1.1, less than 0.9, and more than 1.1 indicate additivity, synergy and antagonism, respectively [38]. Owing to different mechanisms of action, we might expect that the combination of chemotherapy and oHSV would work additively or synergistically. However, it should be noted that combinations could also act antagonistically, although this is rarely published [35,41]. This could be due to chemotherapy-induced early apoptosis or other cellular alterations that directly inhibit the virus life cycle or virus replication. There have been a number of studies using a variety of different drug–oHSV combinations in different cancer cell types, which are summarized in Table 2.

A number of different mechanisms underlying increased efficacy associated with synergistic interactions have been described. In order to achieve effective or synergistic interactions, it is generally beneficial if the chemotherapy does not interfere with the replication of oHSV in infected tumor cells. Indeed, enhancement of oHSV replication by chemotherapy constitutes an important mechanism behind increased tumor cell killing. Increased cytotoxicity has also been found when one agent affects the other in a positive fashion without an increase in oHSV replication. Depending upon the drug mechanism of action, the sequence of administration of the two agents can be critical for a positive interaction, for example, inducing a beneficial cellular environment for the virus or, conversely, if the drug directly interacts with the virus in a negative fashion. Since oncolytic strategy is based on virus replication and spread

throughout the tumor, HSV genes sensitizing cells to chemotherapeutic drugs that accelerate cell death can be detrimental to therapy if they inhibit the virus lifecycle. Alternatively, when the virus expresses genes that block cell death pathways, such as apoptosis [42], the combination can also be counterproductive [43]. However, mutants in genes that block apoptosis, such as HSV *Us3*, can sensitize cancer cells to chemotherapy, as seen with L1BR1 in combination with CDDP or 5-FU, where the proportion of apoptotic cells was significantly increased compared with drug alone or wild-type HSV/drug combination [44]. Drugs that can be used in combination with oHSV are not limited to chemotherapeutics with direct cytotoxic properties. Modulation of tumor microenvironments, physical barriers, such as the extracellular matrix and interstitial pressure, and angiogenesis represent other therapeutic targets to achieve enhanced oHSV-mediated antitumor therapy [45–47]. In this article, we will focus on combinations with:

- Standard-of-care chemotherapies;
- Emerging pharmacological agents;
- Prodrug-activating gene expression.

Combination with ‘standard-of-care’ chemotherapies

For most cancers, standard treatment protocols include chemotherapeutic drugs. However, currently used doses are frequently associated with, and limited by, a variety of adverse side effects that include bone marrow suppression and hepatic-renal-gastrointestinal toxicity. Therefore, it is both reasonable and practical to use oHSV in addition to ‘standard-of-care’ drugs. Preclinical studies both *in vitro* and *in vivo* are critical to determining whether the interaction between the two agents is beneficial and ensuring safety before clinical translation. When synergistic interactions in cancer cell killing are observed, chemotherapy dose reductions that achieve the same overall efficacy may be possible, resulting in a reduction of adverse side effects. This can be especially beneficial for patients, and thus provides a further rationale for the combination strategy.

In a prototypical study by Toyoizumi *et al.*, the combinations of oHSV 1716 and any of four standard chemotherapeutic drugs (MMC, CDDP, methotrexate and ADR) were tested for efficacy against five human NSCLC lines [48]. The interactions of the combined agents were evaluated using isobologram analysis. For two out of five NSCLC lines, the combination of 1716 and MMC worked synergistically, while it was additive for the remaining three cell lines. MMC did not affect viral replication at the doses used, and 1716 did not affect MMC metabolism [48]. Interestingly, CDDP, methotrexate or ADR with 1716 were only additive, suggesting that a general induction of apoptosis, common to these cytotoxic drugs, was not responsible for the synergy but rather distinct drug-related responses. These studies were the first to demonstrate that chemotherapy and oHSV could act synergistically to kill cancer cells, and that this would enhance their antitumor activity. More recent studies have begun to examine the mechanisms underlying the positive interactions between oHSV and chemotherapeutic drugs, and will be discussed later.

Enhancement of virus replication by chemotherapeutic drugs

Early-generation oHSVs (e.g., hrR3 and 1716) were constructed by introducing deletions or mutations into wild-type HSV-1, thereby increasing safety and tumor selectivity. The mutations/deletions, respectively, were in:

- *UL39*, which encodes ICP6, the large subunit of viral ribonucleotide reductase (RR);
- γ*34.5*, whose product protein functions as a major virulence factor, inhibits autophagy, and blocks virus-induced host protein shutoff due to signaling through the interferon

and stress pathways by binding to protein phosphatase I and dephosphorylating translation factor eIF2α [49].

The viral mutants can efficiently replicate in dividing tumor cells because there are cellular homologs that can compensate (e.g., cellular RR and growth arrest-DNA-damage protein 34 [GADD34] for γ34.5) or because the cellular pathways are inactive. Hence, chemotherapeutic drugs that upregulate the expression or activities of these homologs in tumor cells, such as through DNA-damage responses, might enhance oHSV DNA replication of appropriate mutants, leading to enhanced tumor cell killing.

Nucleoside analogs

The mechanism of drug induction of cellular homologs leading to enhanced virus replication was first demonstrated by Petrowsky *et al*., using fluorodeoxyuridine (FUdR, floxuridine), a pyrimidine analog inhibitor of thymidylate synthetase used to treat colorectal cancer, which causes nucleotide pool imbalances and DNA damage. FUdR increased G207 replication in colorectal cancer cell lines, while it decreased the virus yield of wild-type HSV, although this was still at a much higher level than with G207 [50]. This was due to the upregulation of cellular RR, which overcame the negative effect of thymidylate synthetase inhibition for G207 but not wild-type HSV [50]. GADD34 was also induced in the FUdR-treated sensitive cells, but only at higher doses, suggesting that it was not a key contributor [50]. In gallbladder and gastric cancer cells, another nucleoside analog, 5-FU, was found to increase G207 replication *in vitro* and extend survival in peritoneal tumor models [51]. This correlated with increased RR activity and virus spread in the tumors after 5-FU treatment [51]. 5-FU and gemcitabine exhibited strong synergy in combination with NV1066 in pancreatic cancer, which was accompanied by an increase in virus replication [52]. Since NV1066 does not have a RR mutation, GADD34 expression was examined and found to be elevated after 5-FU treatment in pancreatic cancer cells [52]. The direct role of this is unclear because NV1066 retains a copy of γ*34.5* and should be less affected by GADD34 levels. In studies with NV1020 and 5-FU, no increase in virus replication was observed [34], and this will be discussed in the following section (‘Enhancement of chemotherapeutic cytotoxicity by oHSV’).

Mitomycin C

Upregulation of GADD34 expression is a mechanism to enhance the replication of γ*34.5*-mutant oHSV. MMC treatment of gastric cancer cells increased G207 replication, contributing to synergistic killing by MMC and G207, and upregulated GADD34 [53]. When GADD34 was knocked down by GADD34 siRNA, virus replication and cytotoxicity in combination with MMC were greatly diminished [53]. This demonstrates a role for GADD34 upregulation in the synergistic interaction of these two agents. MMC was also shown by this group to synergize with NV1066 in bladder cancer cells, but there was no increase in virus replication [54].

Temozolomide

The combination of TMZ, the first-line alkylating chemotherapy for malignant gliomas worldwide, and G207 was shown to work synergistically to kill TMZ-sensitive, methyl-guanine methyltransferase (MGMT)-negative human glioma cell line U87, due to upregulation of GADD34 by TMZ [35]. No synergy was observed with wild-type HSV. For TMZ-resistant MGMT-positive glioma cells (T98 or U87-MGMT), the interaction was only synergistic in the presence of an MGMT suicide substrate, O^6-benzylguanine, and this was due to upregulation of RR. Thus, the induction of different repair pathways in response to alkylation has effects on oHSV with different mutations (γ*34.5* and ICP6). In both cases, siRNA knockdown of GADD34 and RR inhibited the synergy and increased TMZ-induced DNA damage, respectively [35]. The TMZ/G207 combination targeted tumor cells escaping TMZ toxicity, such that those cells with decreased double-strand breaks and increased GADD34 expression

were most susceptible to G207 replication. Importantly, from a safety standpoint, the combination worked antagonistically in normal human astrocytes *in vitro*. *In vivo*, the TMZ/G207 combination completely eradicated U87 tumors established in the brains of nude mice, while either monotherapy could only provide a survival advantage over mock treatment animals [35]. In addition to the effects of TMZ on virus replication, it has been shown that the combination of TMZ with replication-deficient HSV vectors expressing ICP0 enhances therapy *in vivo*, possibly due to ICP0 inhibiting DNA repair [55].

Cisplatin

In malignant pleural mesothelioma cells, CDDP, part of the standard chemotherapeutic regimen [56], and NV1066 interacted in a synergistic fashion, so that in seven malignant pleural mesothelioma cell lines, CDDP doses could be decreased from three- to 600-fold and NV1066 from 1.4- to seven-fold at 50% of the lethal dose [57]. This synergy in VAMT cells correlated with increased viral replication and induction of GADD34. Again, treatment with siRNA to GADD34 greatly reduced CDDP induction of GADD34 mRNA and protein, as well as synergistic cell killing [57]. This was somewhat unexpected because NV1066 retains a copy of *γ34.5*. The effect of *γ34.5* copy number needs to be further explored. In other systems, CDDP did not increase oHSV replication, and this will be discussed in the following section ('Enhancement of chemotherapeutic cytotoxicity by oHSV').

In summary, the replication of oHSV with *γ34.5* or *UL39* mutations can be enhanced if chemotherapy upregulates the expression or activity of GADD34 or RR, respectively, in tumor cells. This often results in synergistic cytotoxicity and increased therapeutic efficacy. These combinations are beneficial because of the mutations present in the oHSV, and, therefore, are selective for particular oHSVs and should not enhance wild-type HSV, as has been reported for FUdR [50] and TMZ [35]. One concern associated with chemotherapy-enhanced oHSV replication is that it may hamper the safety profile of oHSVs *in vivo*. Therefore, it will be important to examine safety issues in detail, as well as to validate the efficacy of these combinations in additional models.

Enhancement of chemotherapeutic cytotoxicity by oHSV

As described earlier, the ability of certain chemotherapeutic agents to alter tumor cell physiology so that oHSV replicates more efficiently, thereby producing larger virus yields, is an important mechanism underlying synergy. However, there are other mechanisms by which synergistic interactions can be generated, for example, when oHSV infection alters the tumor cell so that chemotherapy has enhanced toxicity through the induction of drug-metabolizing enzymes, apoptosis or autophagy pathways or cell cycle alterations.

Taxanes

In the study by Lin *et al.*, G207 and paclitaxel, a microtubule stabilizer, were shown to work synergistically to kill anaplastic thyroid cancer cells [58]. Paclitaxel did not enhance G207 replication, virus entry or early gene expression, but G207 infection enhanced paclitaxel-induced anticancer activities, microtubule acetylation, mitotic block and apoptosis [58]. Therefore, G207 infection probably augmented and/or lowered the dose threshold for paclitaxel-induced tumoricidal activity, leading to the observed synergistic killing. In a separate study, the taxanes paclitaxel or docetaxel in combination with G47Δ were used to treat prostate cancer [59]. Synergistic killing of prostate cancer cells was noted without an increase in G47Δ replication. The explanation for the observed synergy was that oHSV and taxanes differentially affected cell cycle progression, either by arresting cells at G1 or mitosis, respectively, and increased apoptosis. When prostate cancer cells were treated with both agents, a certain proportion of cells exited the mitotic checkpoint prematurely, resulting in enhanced

prostate cancer cell killing [59]. Inhibition of G47Δ replication with acyclovir abrogated the combination effects on mitosis. In U87 glioma cells, no synergy was observed with docetaxel and G47Δ [Wakimoto H, Unpublished Data]. The combination of HF10, an oHSV currently in clinical trials, with paclitaxel was examined in murine colon cancer CT26 cells *in vitro* and *in vivo* [60]. The combination was more efficacious than each agent alone and did not enhance virus replication, although the mechanism of enhanced efficacy was not explored. Another microtubule inhibitor, vincristine, enhanced G207 cytotoxicity in human rhabdomyosarcoma cells without affecting virus replication [61]. Intravenous treatment with G207 and vincristine in mice with rhabdomyosarcoma xenografts resulted in 60% cures versus none with either agent alone [61]. We found no synergy between vincristine and G47Δ in U87 glioma cells [Wakimoto H, Unpublished Data]

5-FU

In studies with NV1020 and 5-FU in human colon cancer cells, the combination was additive to synergistic for cytotoxicity (HT-29 cells) and synergistic in an *in vitro* clonogenic assay (WiDr cells). However, in this case, virus yield was actually reduced approximately tenfold in the presence of 5-FU [34]. In a syngeneic tumor model (CT26), with three injections of NV1020 followed by three treatments of 5-FU, the combination was significantly better at inhibiting tumor growth and prolonging survival than either treatment alone [34]. In the CT26 model, the immune system plays a large role in oHSV-induced inhibition of tumor growth [62]. The impact of 5-FU on the immune response to oHSV-infected tumors has not been explored. The authors speculate that synergy, in the presence of reduced virus replication, could be due to immune cell expression of death-inducing molecules such as TRAIL and/or IFN-γ and Fas/Fas ligand, which have been shown to enhance cell death induced by 5-FU [63,64]. In this regard, 5-FU has been used to enhance the activity of oncolytic adenovirus ONYX-015 expressing TRAIL [65]. This illustrates a further twist to the combinatorial strategy, where the OV expresses a factor that acts to enhance the cytotoxicity of a drug. A similar strategy is to have the OV express a prodrug-activating enzyme to increase drug metabolism to its active component in the tumor (see later section).

Cisplatin

In glioma cells (U87), CDDP was synergistic with G207; however, there was no induction of GADD34 expression [35]. In an analysis of human head-and-neck SCC cells, CDDP in combination with 1716 was found to have an additive, rather than synergistic, effect, with no enhancement of viral replication [66]. The combination of oxaliplatin with NV1020 was found to be additive to synergistic in human colon carcinoma cells, irrespective of whether the drug was provided before or after the virus [34]. In an *in vivo* model with CDDP-sensitive human head-and-neck SCC cells (UMSSC-38), the combination of G207 and CDDP was significantly better than either of the treatments alone, shrinking tumors and leading to cures in all mice [33]. These investigators did not evaluate *in vitro* interactions, while the other researchers did not evaluate *in vivo* efficacy, and none addressed the mechanism.

Topoisomerase inhibitors

SN38 (the active metabolite of irinotecan, a topoisomerase I inhibitor) in combination with NV1020 *in vitro* was found to work additively and synergistically in a panel of human colon carcinoma cell lines [34]. By contrast, we found that SN38 did not synergize with G47Δ in killing glioma cells (U87 and T98G), but rather was antagonistic at EC_{50} or higher, whether SN38 was added with, before or after G47Δ in U87 cells. No significant change in virus yield was observed with SN38, but at concentrations greater than the EC_{50}, SN38 reduced plaque size on Vero cells [Cheema T, Rabkin SD, Unpublished Data]

Combination with other pharmacological agents

Recent research has expanded the search for potential pharmaceuticals to enhance oHSV therapy, and the candidates are no longer limited to conventional chemotherapeutic drugs. These include molecular-targeting small molecules and signaling pathway kinase inhibitors.

Histone deacetylase inhibitors

One such class is the histone deacetylase inhibitors (HDACi), which have pleiotropic effects on cells through the inhibition of deacetylation of proteins, including histones that then alter the epigenome and transcription. There are 11 zinc-dependent HDACs, which have been the target of drug discovery for cancer therapy, either alone or in combination with chemotherapeutic agents [67,68]. Valproic acid (VPA), a HDACi widely used as an antiepileptic drug, greatly increased the virus yield of MGH2 and rQNestin34.5 and their cytotoxicity in human glioma cells *in vitro* when added before the virus, but not concurrently [69]. This effect was due to inhibiting type 1 interferon responses that inhibit viral gene expression and replication. VPA alone was not cytotoxic to two out of three human glioma cell lines, and analysis of synergy was not reported. The combination also increased rQNestin34.5 yield *in vivo* and extended the survival of mice bearing intracerebral tumors [69]. Interferon responses and innate immunity play a critical role in host defense mechanisms against viruses, and this study illustrates how targeting these responses can enhance oHSV therapy. Similar effects have also been described for oncolytic vesicular stomatitis virus and suberoylanilide hydroxamic acid (vorinostat), a HDACi that has been approved for clinical use [70]. Interestingly, VPA was shown to antagonize oncolytic adenovirus efficacy by inhibiting adenovirus replication late in the viral life cycle [71].

Trichostatin A (TSA), another HDACi, synergizes with G47Δ to promote killing of a broad range of cancer cells and proliferating endothelial cells, but not quiescent endothelial cells or normal prostate cells [72]. This occurs irrespective of the dosing sequence of TSA and G47Δ and does not alter virus infectivity or replication. Similar synergy was also seen with wild-type HSV [72]. The mechanism of synergy was explained by downregulation of cyclin D1 in cells with high levels of cyclin D1, for example cancer cells [72]. The combination also enhanced the inhibition of VEGF secretion from glioma cells *in vitro*, which correlated with a decrease in microvessel density *in vivo* [72]. TSA in combination with R849, another γ*34.5* mutant, enhanced cytotoxicity in SCC cells and was reported to increase oHSV replication, but only at 24 h post-infection, not 12 or 36 h [73]. The mechanism was proposed to be due to activation of NF-κB and cell cycle arrest at G1, although the effects of TSA alone were not significantly different to those of the combination [73].

Molecular-targeting drugs

Recently, small-molecule inhibitors specifically targeting oncogenic signaling pathways have been emerging as effective anticancer agents. The PI3K-Akt signaling pathway is central to the survival of many tumors and cancer stem cells (CSCs) [74]. It has been shown that Akt activation is increased after infection by HSV-1 *Us3* mutants [75,76], and that *Us3*-mutants have oncolytic activity [44,76]. Therefore, it was reasonable to test whether PI3K/Akt inhibitors could synergize with R7041 in killing cancer cells. PI3K inhibitor LY294002 and Akt inhibitor IV were both synergistic with R7041, but not with wild-type HSV *in vitro*, and the combination was significantly better than the individual agents at inhibiting tumor growth [76]. Interestingly, LY294002 inhibits the replication of γ*34.5*Δ oHSV R3616 in pancreatic cancer cells owing to dysregulated PI3K overcoming/complementing the loss of γ*34.5* [77].

Receptor tyrosine kinase inhibitors

Another class of molecular targeting drugs is the receptor tyrosine kinase inhibitors. Erlotinib, an EGF receptor inhibitor, was found to enhance the cytotoxicity of G207 and hrR3 in human malignant peripheral nerve sheath tumor cells *in vitro*, but had no additional effect on tumor growth inhibition *in vivo* [78]. As most molecularly targeted drugs are often ineffective as monotherapy, the combination with oHSV provides an additional potential avenue for their use.

Thalidomide

Thalidomide was approved by the US FDA to treat newly diagnosed multiple myeloma patients, and is being clinically tested in combination with other anticancer drugs [79]. It was investigated in conjunction with OncdSyn in a murine syngeneic mammary carcinoma model. Either thalidomide or OncdSyn alone was effective in reducing tumor burden, but the combination resulted in an additional significant benefit [80]. The mechanism of action is unclear, but thalidomide did not inhibit tumor or endothelial cell proliferation, and the combination did not alter the cytokine profile of splenocytes isolated from treated mice [80].

Prodrug-activating oHSV vectors to enhance chemotherapy

Many chemotherapeutic drugs are converted to active agents by metabolism, usually in the liver. Therefore, to enhance the local concentration of active metabolites in the tumor, oHSVs, as well as replication-deficient viral vectors expressing prodrug-activating enzymes, have been used. This has also been referred to as gene-directed enzyme prodrug therapy [81]. This strategy is appealing for replication-deficient vectors because of potential bystander effects, owing to the ability of the active metabolites to mediate the toxicity of neighboring nontransduced cells. Even for oHSV, this strategy has gained popularity because enhanced overall efficacy has been observed [82,83]. The classic ‘suicide gene’ strategy uses the *HSV-TK* gene to convert ganciclovir (GCV) to its toxic substrate, GCV-triphosphate. While thymidine kinase sensitizes cells to GCV, it can be counterproductive for oHSV because it inhibits viral replication prematurely [84], unless there is a large bystander effect [85]. Other prodrug-activating enzymes that have been utilized with oHSVs are: rat cytochrome P450 (CYP2B1) for the conversion of cyclophosphamide (CPA) to phosphoramide mustard and acrolein [82]; bacterial cytosine deaminase for the conversion of 5-fluorocytosine to 5-FU [86], human or rabbit carboxylesterase for the conversion of irinote-can (CPT-11) and paclitaxel-2′-ethylcarbonate to SN38 and paclitaxel, respectively [87,88]; and bacterial nitroreductase for the conversion of CB1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide) to a potent DNA-crosslinking 4-hydroxylamine [83]. Of these, CPA [89,90], 5-FU and paclitaxel have been examined in combination with oHSV in the absence of activating enzyme expression. However, the combination with oHSV expressing the activating enzyme resulted in increased antitumor efficacy *in vivo* [83,86]. oHSV rRp450, carrying the *CYP2B1* transgene, exhibited enhanced efficacy in combination with CPA in suppressing the growth of subcutaneous glial tumors and diffuse liver tumors in rodents [82,91]. With safety demonstrated in preclinical studies [92], rRp450 is moving towards clinical trial.

Conclusion

Preclinical studies have shown that oHSVs can synergize with a variety of agents, including conventional chemotherapeutics, molecular targeting agents and small-molecule inhibitors. The combination effects often depend on the genetic makeup of the oHSV, the drug used and the cancer type examined. Synergy is not always necessary for clinical translation; even additive effects can be beneficial, especially if the effective chemotherapeutic dose can be reduced to decrease drug-related toxicities, and increasing efficacy by even a small amount

can potentially have a large impact on quality of life and survival. Since oHSVs have relatively limited adverse effects, the risks associated with combining therapy should be minimal unless antagonistic interactions occur *in vivo*. Those drugs that induce DNA repair are likely to combine most effectively with oHSV mutants that are complemented by DNA-damage-inducible genes, such as ICP6^{-} and γ*34.5*$^{-}$ [1]. For circumstances where chemotherapy induces cell death pathways that can be inhibited by HSV, such as apoptosis (*Us3*) [42] or autophagy (γ*34.5*) [93], mutations in those genes should enhance combination therapy. There are combinations that enhance chemotherapeutic cytotoxicity or sensitize cells that are due to the expression, rather than loss, of HSV genes (i.e., *ICP0* and *TK*), and in these cases, synergy should not be dependent upon viral mutations.

It should be noted that the interaction determined *in vitro* might not always parallel the efficacy seen *in vivo*. Determinations of synergy *in vivo* are typically not performed and require large numbers of animals owing to additional difficulties in controlling for variables such as pharmacokinetics, pharmacodynamics, greater measurement variability and multiple, often unknown, targets. In the *in vivo* tumor context, there are likely to be additional interactions and mechanisms of action that go beyond tumor cell cytotoxicity, including enhanced or diminished activity of the immune system, effects on the tumor microenvironment (i.e., stromal cells and the extracellular matrix) and angiogenesis, expression or repression of secreted factors (i.e., cytokines and chemokines), and toxicity to normal cells and organs. Moreover, synergy observed in one drug–oHSV combination in one particular cancer type (or even in one cell line) cannot always be generalized to other drug–oHSV combinations or to different cancer types. For example, paclitaxel was additive to synergistic with NV1023 in thyroid cancer cells, while its combination with adriamycin was antagonistic [58], and TMZ was synergistic with G207 in MGMT-negative glioma cells but was additive to antagonistic in MGMT-positive glioma cells [35]. An especially important facet of these studies that has been relatively ignored so far, especially if this strategy is to be translated to the clinic, is the safety consequences of combination therapy. It will be necessary to determine whether the drug–oHSV combinations reveal new toxicities or decreased safety profiles compared with what is observed when they are administered individually.

Major progress and some surprises are likely to occur as we translate these preclinical studies into clinical trials. Multimodal treatment strategies are the mainstay of cancer therapy, and oHSV is unlikely to behave differently. It is only through testing the combinations in patients that we will determine whether this strategy will impact cancer therapy. As many of the drug combinations described here are with standard-of-care chemotherapeutics, some clinical trials with oHSV have invariably included combination treatments, although they have not been specifically dosed for that purpose. For example, in a Phase I clinical trial with NV1020 for metastatic colorectal carcinoma to the liver, patients received intra-arterial chemotherapy beginning 28 days after intra-arterial virus infusion. All of the patients had failed first-line chemotherapy, but showed a progressive decrease in carcinoembryonic antigen levels after beginning regional chemotherapy (floxuridine and irinotecan), suggesting that oHSV treatment might have affected the response to chemotherapy [94]. This provides optimism that improvements in treatment outcomes will follow clinical translation of this combinatorial strategy. Any such improvements will be important in advancing oHSVs towards FDA approval.

Future perspective

As has been discussed, the therapeutic outcome of a specific drug–oHSV combination can be different even between cell lines from the same cancer type. This may be caused by tumor cells' varying sensitivity to the drug or oHSV, which may be governed by the expression of multiple and different gene sets. Stratification of tumors into responders and nonresponders to

the combination strategy may provide an opportunity to probe for gene-expression signatures that potentially dictate treatment efficacy. In this context, analysis using gene-expression array may be useful to reveal gene-expression profiles characteristic to responders, which may also lead to discovery of biomarkers predictive of responses to therapy.

The identification of additional drugs that positively interact with oHSV is a high priority. High-throughput screening is a powerful approach to identify new and unexpected compounds in a limited time. In an effort to identify novel compounds that enhance oHSV replication and spread, we have begun screening chemical and drug libraries in a cell-based assay using oHSV expressing green fluorescent protein. Once compounds have been identified and validated, they can be optimized through medicinal chemistry and tested in animal models. Since there are a range of virus mutations that can endow HSV with oncolytic activity and safety, compound screens should include oHSV vectors with different mutations to take advantage of diverse interacting cellular pathways. Understanding how the compound 'hits' enhance oHSV replication and/or cytotoxicity should identify novel cell pathways, and these might be targetable by already developed or approved drugs.

The field of siRNA-based therapy offers great promise for personalized treatment of cancer, including targets, such as oncogenes and genes, that are involved in angiogenesis, metastasis, survival, anti-apoptosis and resistance to chemotherapy [95]. Combining the siRNA platform with oHSV may also improve efficacy, since it will allow for the specific knockdown of genes of interest that may be overexpressed in tumors. Another newly emerging field in cancer biology is the role of miRNA [96]. Delivery of miRNAs or miRNA inhibitors is likely to be a novel combinatorial strategy.

Increasing evidence suggests that there exists a small population of tumor cells that are responsible for the recurrence and maintenance of cancer. These cancer-initiating cells or CSCs have been reported to be more resistant than other cells to conventional treatment modalities, such as radiotherapy [97]. There is extensive research ongoing to develop novel therapeutics that target the CSC population, as opposed to the bulk of the tumor mass, which had been the focus of drug discovery during the preceding decades. CSCs have been shown to be sensitive to killing by oHSVs [98–100]. Therefore, examining oHSV combinations with the drugs described in this article, as well as novel drugs targeting CSCs, will be an important avenue for enhancing cancer therapy.

Most of the described studies were initiated by examining tumor cells *in vitro*; however, many interactions will affect tumor or host biology in addition to cancer cell cytotoxicity and will need to be examined *in vivo*. One important area is the contribution of the immune response, such as chemotherapy-induced 'immunogenic cancer cell death' [101], and how this could be augmented with immunomodulatory factors. Conversely, innate immune effects can limit the effectiveness of oHSV therapy [15]. CPA and HDACi block these acute responses, and additional agents should be explored, as well as evaluating whether blocking innate immunity contributes to any of the improved treatment outcomes seen with other drugs discussed in this article. Metronomic chemotherapy dosing is gaining support for cancer treatment, and combining metronomic drug dosing with oHSV may prove beneficial. Anti-angiogenesis is one of the therapeutic consequences of metronomic chemotherapy, and it is likely that anti-angiogenic or vascular normalization compounds will improve combination strategies. All in all, there is much that remains to be explored in developing drug–oHSV combination therapy.

Executive summary

Oncolytic herpes simplex virus–drug combinations

- Chemotherapy can synergize with oncolytic herpes simplex virus (oHSV) to kill cancer cells *in vitro*.

Combination with 'standard-of-care' chemotherapies

- Drug–oHSV combinations can enhance the inhibition of tumor growth and extend the survival of tumor-bearing mice compared with monotherapy.
- There is a broad range of drugs that can positively interact with oHSV to improve efficacy.

Enhancement of virus replication by chemotherapeutic drugs

- Combinations can synergize owing to increased oHSV replication and virus yield, but this is dependent on the particular oHSV mutation and chemotherapy-induced DNA-damage response.

Enhancement of chemotherapeutic cytotoxicity by oHSV

- oHSV can sensitize cancer cells to chemotherapy.
- Just as chemotherapeutic drugs vary in their efficacy in different tumor types, outcomes with their combination with oHSV will also differ depending on the cancer cell.

Prodrug-activating oHSV vectors to enhance chemotherapy

- 'Armed' oHSV expressing prodrug-activating enzymes can produce high local concentrations of toxic metabolites, which increases efficacy.

Conclusion

- Since many of the effective drug–oHSV combinations include standard-of-care drugs, they should be readily translatable to the clinic.

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▪ of interest

▪▪ of considerable interest

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Table 1

Oncolytic herpes simplex virus vectors.

oHSV	Parental strain	Mutated/deleted HSV genes	Transgenes/inserted genes	Disease	Ref.
oHSV vectors in clinical trials					
G207	F/R3616	γ*34.5* and ICP6	*LacZ*	Recurrent glioma	[10]
1716	17+	γ*34.5*	*N*	Glioma and neuroblastoma	[102]
OncoVEX	JS1	γ*34.5* and *α47*	*GM-CSF*	Melanoma and pancreatic cancer	[103]
NV1020	F	U_L*24* and U_L*56*	*HSV-2 gG, gJ, gD* and *gI*	Colorectal cancer	[104]
HF10†	HF	U_L*56*†	*N*	Breast cancer and pancreatic cancer	[105]
G47Δ	F	γ*34.5*, ICP6 and *α47*	*LacZ*	Recurrent glioma	[106]
Other oHSV vectors combined with chemotherapy in preclinical studies					
R3616	F	γ*34.5*	*N*		[107]
hrR3	KOS	ICP6	*LacZ*		[108]
rRp450	KOS/hrR3	ICP6	*CYP2B1*		[82]
MGH2	F/R3616	γ*34.5* and ICP6	*CYP2B1*, carboxylesterase and *GFP*		[87]
rHsvQ1	F/R3616	γ*34.5* and ICP6	*GFP*		[109]
NV1066	F	Single copy of ICP0, ICP4 and γ*34.5*	*GFP*		[110]
rQNestin34.5	F/R3616	Endogenous γ*34.5*, ICP6	Nestin promoter-driven γ*34.5*		[109]
R7041	F	*Us3*	*N*		[111]
R849	F	γ*34.5*	*LacZ*		[112]
OncdSyn	F/NV1020	U_L*24* and U_L*56*	*HSV-2 gG, gJ, gD, gI, gBsyn3* and *gKsynI*		[113]

†Clonally selected virus.

CYP: Cytochrome P450; GFP: Green fluorescent protein; N: None; oHSV: Oncolytic herpes simplex virus.

Table 2

Preclinical studies utilizing drug and oncolytic herpes simplex combinations.

Drug	oHSV	Cancer type	Cell line	Interaction *in vitro*	*In vivo* efficacy	Comments	Ref.
Mitomycin C	1716	NSCLC	NCI-H460 and A549	S	+ (NCI-H460)	NA	[48]
			Calu-1, Calu-3 and NCI-H322	A	ND		
	G207	Gastric cancer	OCUM-2MD3	S	+	E	[53]
			MKN-45-P	S	ND		
	NV1066	Bladder transitional	KU19-19	S	ND		[54]
		Carcinoma	SKUB	S	ND		
5-fluorouracil	G207	Gallbladder cancer	KIGB-5 (murine)	ND	+	E	[51]
		Gastric cancer	MKN45 (human)	ND	+		
	NV1020	Colon cancer (human and murine)	HT29	A to S	ND	NA	[34]
			WiDr	A to S	ND	The interaction was not affected by the sequence of administration	
			HCT-116	A to S	ND		
			CT-26		+		
	NV1066	Pancreatic cancer	Hs 700T	S	ND	E	[52]
			PANC-1 and PaCa-2	S	ND		
Fluorodeoxyuridine	G207	Colon cancer	HCT8	ND	ND	E	[50]
			HCT8/FU7dR	ND	ND		
Gemcitabine	NV1066	Pancreatic cancer	Hs 700T	S	ND	E	[52]
			PANC-1 and PaCa-2	S	ND		
	R3616	Pancreatic cancer	CAPAN1 and PaCa-2	ND	+ (both cell lines)	D	[41]
	hrR3		SW1990	ND	−	D	
Methotrexate	1716	NSCLC	NCI-H460	A	ND		[48]
Cisplatin	1716	NSCLC	NCI-H460	A	ND		[48]
		Head-and-neck SCC	Three cell lines	A	ND	Cisplatin was not toxic to 1716	[66]

Drug	oHSV	Cancer type	Cell line	Interaction *in vitro*	*In vivo* efficacy	Comments	Ref.
	NV1066	Malignant pleural mesothelioma	Ten cell lines	S in seven cell lines, A in the three others	ND	Increased viral replication in one cell line. Synergy was eliminated by siRNA to GADD34	[57]
	G207	Head-and-neck SCC	UM-SCC-38	ND	+	Cisplatin was not toxic to 207 (SCC-25/CP)	[33]
			SQ20B and UM-SCC 22A		− (SQ20B)		
			SSC-25/CP and PCI51		ND		
	G47Δ	Prostate cancer	LNCaP	ANT	ND		[59]
Oxaliplatin	NV1020	Colon cancer	HT29 and WiDr	A to S	ND	The interaction was not affected by the sequence of administration	[34]
			HCT-116	A to S	ND		
Doxorubicin	1716	NSCLC	NCI-H460	A	ND		[48]
	G47Δ	Prostate cancer	LNCaP	ANT	ND		[59]
Temozolomide	G207	Malignant glioma	U87	S	+	E	[35]
			U87-dnp53 and U373	S	ND		
			T98 and U87MGMT	S (O^6-BG)	ND		
Vincristine	G207	Rhabdomyosarcomas	RD, Rh1, Rh28 and Rh30	ND	ND	NA	[61]
			Rh41, CCA and KFR		+ (KFR)		
			KF-RMS-1		+		
Paclitaxel	G207	Anaplastic thyroid cancer	KAT4	S	+	NA	[58]
			DRO90-1	S	ND	Mechanisms of paclitaxel antitumor activity were enhanced by G207	
	G47Δ	Prostate cancer	LNCaP	S	ND	NA	[59]
			DU145	S	ND		
	MGH2	Mammary carcinoma	MDA-MB-435S	ND	+	oHSV spread was enhanced due to void spaces created by the drugs Sequence of administration critical	[114]

Drug	oHSV	Cancer type	Cell line	Interaction *in vitro*	*In vivo* efficacy	Comments	Ref.
			MDA-MB-361HK	ND	ND		
	HF10	Colon cancer (murine)	CT26	ND	+	NA Combination was more efficacious than single agents alone *in vivo*	[60]
Docetaxel	G47Δ	Prostate cancer	LNCaP	S	+	NA	[59]
			DU145	S	ND		
Etoposide	G47Δ	Prostate cancer	LNCaP	ANT	ND		[59]
Irinotecan (SN38)	NV1020	Colon cancer (human and murine)	HT29 and WiDr	A to S	ND	The interaction was not affected by the sequence of administration	[34]
			HCT-116	A to S	ND		
Valproic acid	rQNestin34.5	Glioma	U251 and U87Δ EGFR	ND	+ (U87Δ EGFR)	E (when valproic acid was administered prior to oHSVs)	[69]
	rHsvQ1		Gli 36Δ EGFR and OHG02†	ND	ND		
Trichostatin A	G47Δ	Glioma	U87 and T98	S	+ (U87)	NA	[72]
		Colon cancer	SW480	S	+		
		Cervical cancer	HeLa	S	ND		
		Breast cancer	MCF-7	A	ND		
	R849	Oral SCC	SAS, Ca9-22 and HSC	ND	ND	E (SAS)	[73]
Thalidomide	OncdSyn	Mammary carcinoma (murine)	4T1	ND	+	Combination was better at controlling the growth of established tumors than single agents	[80]
LY294002	R7041	Glioma	U87	S	+		[76]
Akt inhibitor IV		NSCLC	A549	S	ND		
Erlotinib	G207	MPNST		A	−		[78]

Drug	oHSV	Cancer type	Cell line	Interaction *in vitro*	*In vivo* efficacy	Comments	Ref.
Cilengitide	hrR3, rHSVQ1 rQNestin34.5	Glioma (human and murine)	D74HveC	ND	ND	Cilengitide blocked proangiogenic effect of oHSVs	[46]

†Primary cultured human glioma cells.

+Overall efficacy was augmented by chemotherapy *in vivo*; −: overall efficacy was not augmented by chemotherapy *in vivo*; A: Additive; ANT: Antagonistic; D: Viral replication was decreased by chemotherapy; E: Viral replicatoin was enhanced by chemotherapy; EGFR: EGF receptor; GADD34: Growth arrest-DNA-damage protein 34; MPNST: Malignant peripheral nerve sheath tumor; NA: Viral replication was not affected by chemotherapy; ND: Not determined; NSCLC: Non-small-cell lung cancer; O^6-BG: In the presence of O^6-benzylguanine; oHSV: Oncolytic herpes simplex virus; S: Synergistic; SCC: Squamous cell carcinoma.