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Bugs and Drugs: Oncolytic Virotherapy in Combination with Chemotherapy

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Abstract

Single agent therapies are rarely successful in treating cancer, particularly at metastatic or end stages, and survival rates with monotherapies alone are generally poor. The combination of multiple therapies to treat cancer has already driven significant improvements in the standard of care treatments for many types of cancers. The first combination treatments exploited for cancer therapy involved the use of several cytotoxic chemotherapy agents. Later, with the development of more targeted agents, the use of novel, less toxic drugs, in combination with the more classic cytotoxic drugs has proven advantageous for certain cancer types. Recently, the combination of oncolytic virotherapy with chemotherapy has shown that the use of these two therapies with very distinct anti-tumor mechanisms may also lead to synergistic interactions that ultimately result in increased therapeutic effects not achievable by either therapy alone. The mechanisms of synergy between oncolytic viruses (OVs) and chemotherapeutic agents are just starting to be elucidated. It is evident, however, that the success of these OV-drug combinations depends greatly on the particular O V, the drug(s) selected, and the cancer type targeted. This review summarizes the different OV-drug combinations investigated to date, including the use of second generation armed OVs, which have been studied with the specific purpose of generating synergistic interactions with particular chemotherapy agents. The known mechanisms of synergy between these OV-drug combinations are also summarized. The importance of further investigating these mechanisms of synergy will be critical in order to maximize the therapeutic efficacy of OV-drug combination therapies in the future.

Keywords

Cancer; chemotherapy; combination therapy; oncolytic virus; synergism; virotherapy

1. INTRODUCTION

Chemotherapy as a cancer treatment was introduced in the late 1940's with the discovery of nitrogen mustards as potential anticancer drugs [1], followed by the discovery of aminopterin, a folic acid antagonist, as a treatment for leukemia that produced regular, albeit

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temporary, remissions [2]. During the 1960s and 1970s the concept of combination chemotherapy and adjuvant chemotherapy was introduced. Regimes involving multiple chemotherapy agents were developed as well as the use of chemotherapy as an adjunct to surgery and radiation [3]. Later, targeted chemotherapy agents with increased specificities in the 1990s were developed. These advances came as dividends of the knowledge gained about the intracellular mechanisms and genetic alterations that initiate and promote tumorigenesis. Even though chemotherapy has improved the standard of treatment for most cancers it has significant limitations and for many cancers still, complete remissions are rare.

Virotherapy as a cancer treatment stemmed from the observation that occasionally, cancer patients would undergo transient remissions after contracting a viral disease [4]. To date, many viruses with oncolytic properties have been identified and are being developed as potential biotherapeutics for cancer. In order to circumvent potential pathogenicity in cancer patients, many of the proposed OVs possess highly attenuated phenotypes (either naturally occurring or engineered). Some of these attenuated OVs were initially developed as vaccines for which clinical grade stocks and extensive safety data of their use in humans is available. Still others have investigated the use of animal viruses that are nonpathogenic in humans as novel OVs [4]. In 2005, after years of extensive preclinical reports and clinical trials, the adenovirus H101 became the first OV to be approved by a regulatory agency for the treatment of human cancers in China [5]. However, the knowledge gained from these reports and clinical trials have also uncovered limitations to effective virotherapy that, like chemotherapy, need to be addressed.

The idea of combining chemotherapy with virotherapy provides an alternative combination treatment regime for cancer [6-8]. In fact, the optimal treatments for complex diseases such as cancer will likely come from the exploitation of sequential therapeutic modalities that enhance or synergize with each other. One strategy aimed at combining virotherapy with chemotherapy is to explore the use of OVs as an adjuvant to standard chemotherapy treatments. This is probably the most clinically relevant strategy, since most cancer patients have been or will be subjected to standard-of-care chemotherapies. Other strategies involve the use of drugs that can enhance virotherapy by counteracting or inhibiting host pathways that limit virotherapy. Regardless of the approach, the use of OVs in combination with drugs has proven in many instances to result in synergistic interactions that improve upon the efficacy of either treatment alone. However, not all OV-drug combination treatments result in an enhanced effect. In many cases, the overall anticancer effects of OV-drug combinations are dependent on the virus strain, the cancer cell type(s) or the exact drugs used. This review will introduce the common problems and limitations of chemotherapy and virotherapy and then will focus on a detailed summary of OV-drug combinations explored to date. Currently, second generation OVs are now being developed which are designed to tackle some of the limitations of OV monotherapy as well as to broaden their applicability for use in combination with specific chemotherapies. Hence, this review will also provide an overview of the different OV-drug model systems aimed at enhancing chemotherapy with armed OVs.

2. CHEMOTHERAPY AND VIROTHERAPY AS SINGLE AGENT THERAPIES

2.1. Chemotherapy

The most common drugs used for cancer chemotherapy are cytotoxic agents designed to "kill" cancer cells resulting in tumor regression and eradication. A wide range of chemotherapy drugs act by inhibiting DNA replication and/or the cell cycle. However, regardless of their mechanism of action, cytotoxic drugs are not generally specific to cancer cells and do not operationally discriminate between normal and cancerous cells, preferentially affecting all rapidly dividing somatic cells. Many organ related toxicities such as cardiac, renal, hepatic, hematological and gastrointestinal are also frequently associated with chemotherapy [9]. In addition to the toxicity and the lack of specificity of these cytotoxic agents, frequently cancer cells may exhibit or develop drug resistance which can be acquired by: 1) impaired membrane transport of drug, resulting in decreased uptake of the drug and/or upregulation of its export, 2) enhanced inactivation of a drug's active metabolites, 3) enhanced DNA repair capabilities, and 4) absence or dysregulation of pathways that lead to cell death in response to DNA damage [10]. A particular problem, which severely compromises the effectiveness of chemotherapy, is the development of a multiple drug resistant (MDR) phenotype. Cancer cells commonly develop MDR by increasing the export of a broad range of anticancer drugs through the cellular membrane, even against concentration gradients [11, 12] .

The increased knowledge of signaling molecules that are commonly affected or dysregulated in cancer cells has lead to the development of novel targeted drugs that specifically target cancer cells and inhibit their growth. These novel targeted drugs are aimed at inhibiting specific signaling pathways commonly dysregulated in cancer cells and that contribute to their transformed phenotype. Still others may inhibit tumor growth by modifying the tumor microenvironment such as the extracellular matrix and tumor vascularization [13]. The advantage of these targeted drugs over classic cytotoxic drugs is their relatively lower toxicity, since they specifically target signaling pathways that are aberrant in cancer cells but not in normal cells. Unfortunately, these targeted drugs in most cases lack effectiveness, particularly over the longer term, and are not as efficient in promoting tumor reduction against complex solid tumors for which cytotoxic drugs remain the most effective [14]. However, due to the complex nature of cancer, the search for effective anticancer therapies should continue to explore as many therapeutic approaches as possible [15].

2.2. Oncolytic Virotherapy

OVs are natural or genetically engineered viruses that exhibit the ability to inhibit tumor progression through various mechanisms. These anti-tumor properties of OVs can include not only the direct infection and oncolysis of cancer cells, but also other indirect mechanisms, such as the preferential activation of the host anti-tumor immunity [16, 17]. The oncolytic potentials of many replication competent and incompetent viruses have been studied. This review will focus on replication competent OVs and their use in combination with chemotherapy agents. Replication competent OVs are very diverse and belong to many different virus families (Table **1**) [18-33].

The molecular basis for the cancer cell tropism of OVs depends on the nature of the virus and/or their specific genetic alterations and these have been reviewed elsewhere [34]. Aberrant intracellular signaling abnormalities in cancer cells can be significant determinants of OV tropism for cancerous cell [35-37]. Other OVs such as Measles virus (MV) rely on the expression of specific cellular receptors for entry and their tropisms can be modified to target specific tumor types [30, 38, 39]. Given these properties, the success of anticancer therapies involving OVs will depend on the biology of the specific cancer cell type targeted and on the specific biological properties of the selected OV.

One of the major advantages of OVs over standard cytotoxic chemotherapy agents is their native tumor cell selectivity enabling them to potentially target and eliminate cancerous cells while sparing non-cancerous tissues [40]. This cancer cell selectivity should make OVs safe and nonpathogenic to patients with minimal or no side effects upon local or systemic administration. To this end, genetically engineered or modified OVs have been developed that improve on the safety of OVs and their specificity for tumor cells [40]. Another potential advantage of OVs is the fact that their oncolytic activity is normally not affected by drug-resistance. In some cases, infection of chemotherapy resistant cells with an OV may make the cells more susceptible to the chemotherapy agent as described later in this review. In addition, OVs can efficiently eliminate cancer stem cells, a tumor cell population that is often insensitive to standard chemotherapies [41, 42] and some, such as herpes simplex virus (HSV) and the Lister strain of vaccinia virus (VACV), can propagate within the hypoxic tumor environment known also to be less responsive to chemotherapy [43, 44]. Given these characteristics, OVs are promising adjuvants for use in combination with chemotherapy agents, particularly for the elimination of metastatic, residual or drug-resistant cancers.

Like chemotherapy, the use of oncolytic virotherapy as a monotherapy for cancer has its limitations. The first major barrier for oncolytic virotherapy is the efficient delivery of the OV to all of the tumor sites. If direct intra-tumoral injection is not feasible due to the tumor's location or in the case of hematological malignancies, the virus must be injected systemically and must somehow efficiently reach all the tumor sites without being detected and cleared prematurely by the immune system. In fact, it is well documented that the host's immune response against the virus is one of the major factors that negatively affects OV therapy by hindering both delivery and spread of the OV [16, 45, 46]. Also, in some cases repeated administration of OVs may be required in order to achieve a therapeutic effect. This repeated dosing may increase neutralizing antibody titers directed against the OV [47] and subsequently reduce the effectiveness of repeated OV doses. In addition, pre-existing immunity to some OVs (*e.g.*, VACV and MV) may also affect their delivery and efficacy. Efficient OV delivery is a nontrivial issue, which is currently being approached by the development of "Trojan horse" or "carrier" cell approaches [48-52]. Also, combination therapies with immunosupressants is another promising alternative to circumvent or at least temporarily suppress anti-viral responses [47, 53, 54]. Aside from enhancing oncolytic virotherapy with cell carriers and immunosuppresants, improvements upon the OVs themselves have led to the engineering of second generation armed OVs that express transgenes aimed at: 1) overcoming the limitations in the spread and oncolysis of OVs, 2)

inducing favorable antitumor immunity, and/or 3) increasing the applicability of these viruses to enhance current standard chemotherapies.

Since 1996, various candidate OVs have reached phase I and II clinical trials. These OVs include specific strains and/or engineered oncolytic HSV-1, reoviruses, adenoviruses, VACV, Newcastle Disease virus (NDV) and MV [55]. However, phase III clinical trials remain in the future for all these viruses except for the adenovirus H101 (Ad-H101), which became the first live virus approved for oncolytic virotherapy of cancer [5]. As with chemotherapy, there is as yet no perfect solution to completely resolve the limitations of oncolytic virotherapy as a monotherapy, but well-managed combination treatment strategies, involving OVs and chemotherapy agents, which complement and enhance each other's effects, may result in successful treatment outcomes for many types of cancer.

3. COMBINATION THERAPY WITH OVS AND DRUGS: MORE THAN THE SUM OF ITS PARTS?

The rationale for the development of novel therapies must consider that cancer is a multifactorial, heterogeneous disease of cells and tissues. In recent years, combination of standard cytotoxic chemotherapy agents with targeted drugs has gained increasing significance in the clinical setting. Unfortunately, not enough of these novel drug combination therapies have significantly improved patient survival when compared to the standard mono-chemotherapy, as in the case of pancreatic adenocarcinoma [56]. For OVs, many preclinical models have shown that combining chemotherapy drugs with OVs may lead to an enhanced therapeutic effect, not only for oncolytic adenoviruses but also for many other OVs. In fact, China approved Ad-H101 in 2005 for the treatment of head and neck cancers after phase III clinical trials showed a higher response rate for Ad-H101 plus chemotherapy with 5-FU (79-72%) compared to chemotherapy alone (40%) [5]. When the right combination of virus and drug are exploited, chemotherapy and virotherapy show great promise in their ability to complement each other and enhance their individual therapeutic effects. Here we discuss ways in which synergy has been accomplished by the use of oncolytic virotherapy with an appropriate chemotherapy. Table **2** provides examples of OV and drug combinations with known mechanisms of synergy.

3.1. Cytotoxic Agents

Many of the current treatments for cancer rely on the use of cytotoxic agents which act primaily by inhibiting DNA replication or by disrupting microtubule structures. Some of these cytotoxic agents have been extensively studied in combination with OVs with the purpose of achieving a better therapeutic outcome or of enhancing the approved standard of care. In clinical trials, attenuated OVs, such as the adenovirus ONYX-015, often have low efficacy as monotherapies, but have favorable interactions when combined with classic cytotoxic chemotherapy agents [57, 58]. In fact, OVs have shown promising synergistic interactions with a wide range of cytotoxic agents.

3.1.1. DNA Crosslinking Agents and OVs

3.1.1.1. Cyclophosphamide (CPA): CPA is delivered as a 'prodrug' that requires intracellular conversion to its active metabolites by cytochrome P450-mediated microsomal oxidation. The anti-tumor effects of CPA are mainly due to its active metabolite, phosphoramide mustard, which crosslinks with DNA, inhibits DNA replication and thereby induces cell death [59]. CPA treatment is typically associated with hematopoietic toxicity, resulting in immunosuppression. Thus, aside from its anti-neoplastic activity, CPA is also considered an immunomodulator as reviewed in [60, 61]. Upon CPA treatment, both humoral and cellular immune responses are inhibited mainly by CPA-induced depletion of B and T cells and by inhibition of their proliferative responses. These CPA induced effects are transient and normal immune responses are usually restored after cessation of treatment [62].

Since one of the major inhibitors of OV replication and spread is the anti-viral immune response, the immunosuppressive functions of CPA have been shown to be critical in inducing synergism with OVs. For example, CPA has been extensively studied in combination with HSV-1. *In vivo*, CPA enhanced initial infection and prolonged replication of systemically administered HSV-1 in rat glioma models. It also produced a synergistic interaction and an overall increase in anticancer efficacy [63, 64]. Similar to HSV-1, the efficacy of systemic delivery of an oncolytic VACV construct that was deleted for two viral virulence genes (called vvDD), was enhanced by CPA in a rat glioma model. CPA treatment also allowed for vvDD from a second dose to infect and replicate in the tumors [65]. Oncolytic virotherapy with other OVs such as HSV-2 [66], reoviruses [67], adenoviruses [68] and MV [69, 70] has also been enhanced by CPA treatment.

The mechanism by which CPA enhances oncolytic virotherapy is believed to occur primarily through the effects that CPA has on the immune system of the host, and not through a direct enhancement of viral replication in the cancer cells. In fact, for HSV-1 and HSV-2, treatment of cells with an active CPA metabolite does not alter virus replication *in vitro* [66, 71]. The CPA mediated enhancement of HSV oncolytic virotherapy involves at least three known mechanisms: 1) a reduction in the levels of preimmune immunoglobulins (Igs) concomitant with a reduction in the activation of complement, 2) the inhibition of local innate antiviral responses within the tumors and, 3) the inhibition of adaptive antiviral immune responses. It has been reported that the activation of complement and the levels of preimmune IgM are important for the clearance of a systemically administered oncolytic HSV-1 derivative, designated hrR3. Upon CPA treatment, IgM plasma levels were reduced. This CPA induced reduction in IgM levels was linked to a reduction in the activation of complement upon systemic injection of HSV-1. With reduced viral clearance by complement, more HSV-1 reached tumors, thereby enhancing the initial infection of tumors and the ability of virus to infect multiple tumors [64]. CPA can also enhance oncolytic virotherapy by inhibiting the function and/or recruitment of innate immune cells that are activated early during OV infection of tumors. During combination therapies with hrR3 and CPA, CPA caused a reduction in the expression of cytokines involved in innate anti-viral immune responses (IFN α/β and γ, tumor necrosis factor [TNF], and interleukin [IL] -15 and -18) [71] and a reduction in the infiltration of macrophages into HSV-1 infected tumors [63].

In vitro, CPA also inhibited the production of IFNγ by NK cells, which are recruited to OV-infected tumors as an early response to infection [63]. A reduction in macrophage infiltration was also reported in combination therapy using the oncolytic VACV, vvDD, and CPA [65]. Finally, CPA has been shown to inhibit or delay the onset of the adaptive immune response against OVs. In several OV and CPA combination therapies, a reduction in the levels of virus neutralizing antibodies (NAb) and/or a delayed production of NAbs compared to OV treatment alone has been reported [64, 67, 69, 72]. Thus, taken together, CPA chemotherapy synergizes with oncolytic virotherapy by inhibiting the innate and adaptive immune responses resulting in prolonged and increased replication of OVs in tumors.

The use of CPA may also allow for a reduction in the OV dose needed to produce a therapeutic benefit thereby reducing any potential or the amount of virus required for efficient therapy [73]. The transient nature of CPA induced immunosupression is also an advantage, since it can allow for a temporal regulation of the immune response which can be restored at an appropriate time to avoid OV related toxicities. In addition, several reports have shown that CPA at appropriate concentrations can deplete regulatory T (Treg) cells, which are associated with tumor-induced immune tolerance and that treatment with CPA may sensitize tumors to immunotherapy [74, 75]. During oncolytic virotherapy, an antitumor response is generated which could be enhanced by CPA-driven depletion of Treg cells. Improved anti-tumor specific responses have been reported with oncolytic HSV-2 or reovirus in combination with CPA [66, 67].

3.1.1.2. Cisplatin: Cisplatin is a platinum based chemotherapy agent that binds to and causes crosslinking of DNA, leading to apoptosis in the cell. Cisplatin was first introduced in clinical trials during the 1970s and showed strong antitumor activity against a wide range of cancer types [59, 76]. Cisplatin has also been reported to synergize with several OVs.

In a murine melanoma model (B16 F10), cisplatin synergized with reovirus by inhibiting the OV-stimulated cytokine and chemokine production, but no effects on the humoral immune response were observed [77]. It was also reported that cisplatin and reovirus synergized in a cancer cell dependent manner among non-small cell lung cancer (NSCLC) cells [78]. VACV has also been reported to synergize with cisplatin in xenografted human pancreatic tumors. When an oncolytic VACV construct deleted for three viral virulence genes, called GLV-1h68, was administrated followed by cisplatin treatment, tumor regression was observed [79]. Also, conditionally replicating oncolytic adenovirus, with deletions that result in attenuated phenotypes or armed with therapeutic genes can also be combined with cisplatin to enhance the overall therapeutic effects in hepatocellular carcinoma [80], head and neck cancer [81, 82], and cervical cancer [83, 84]. Combination of cisplatin and adenoviruses expressing the E1A protein but lacking the E3B gene led to enhanced viral replication in tumor cells and significantly suppressed tumor progression in an immunocompetent tumor model [85]. Cisplatin also enhanced oncolysis of HSV-1 in NSCLC [86], head and neck squamous cell carcinoma [87], and pancreatic cancer models [88]. Cisplatin potentiated the oncolytic effects of NV1066, an oncolytic HSV-1 with a $\gamma_1$34.5 gene deletion, by up-regulating the expression of the growth arrest and DNA damage inducible protein, GADD34, which acted as a functional homolog of the viral $\gamma_1$34.5 in

malignant cells. The synergistic effects between NV1066 and cisplatin allowed for improved viral replication and enhanced cytotoxicity in cancer cells as well as for a reduction in both the virus and drug dose without compromising the oncolytic efficacy [89]. This led to a successful demonstration of synergistic effects using cisplatin and NV1066 in a malignant pleural mesothelioma model where a second-line therapeutic role for oncolytic HSV-1 against chemotherapy and radiotherapy resistant tumors was suggested [90]. Finally, cisplatin synergized with an oncolytic armed VSV by enhancing cytotoxicty in a squamous cell carcinoma model without altering viral replication *in vitro* and without toxicity to normal cells [91]. *In vivo*, cisplatin enhanced the viral replication of an engineered VSV armed with a viral fusion protein after intratumoral delivery, leading to an improvement of the therapeutic effect. However, cisplatin abolished the therapeutic benefit of IL-12 armed VSV suggesting that the side effects of cisplatin on immune cells hindered their response to IL-12 [91].

3.1.2. Nucleoside Analogs and OVs

3.1.2.1. Gemcitabine: Gemcitabine is a cytidine analogue with antitumoral activity against a broad range of solid and hematological cancers. Gemcitabine is delivered as a prodrug that requires cellular uptake by nucleoside trasporters and intracellular phosphorylation to its active metabolites. The triphosphate form of the drug is an inhibitor of DNA polymerase that when incorporated into the elongating DNA strand causes “masked termination”. The diphosphate form of gemcitabine inhibits ribonucleotide reductase (RR), which leads to a self-potentiating effect. By inhibiting RR, the competing cellular deoxyribonucleotide pools are decreased which results in a more efficient phosphorylation and incorporation of gemcitabine into the DNA [92]. Paradoxically, gemcitabine was initially developed as an antiviral agent, but soon after its potent antitumor activities were observed, it was then developed as an anticancer agent. Even though gemcitabine does in some cases inhibit the replication of OVs, the overall antitumor effects *in vivo* are generally enhanced when combination therapies involving these OVs are used.

Several publications have reported synergistic interactions between gemcitabine and adenoviruses. The mechanism for this enhancement is believed to occur through the expression of the adenoviral E1A protein and its effects on cellular factors known to affect sensitivity and resistance to chemotherapy such as nuclear factor-κB (NF-κB) and poly(ADP-ribose) polymerase (PARP) [93][94, 95]. In hepatocellular carcinoma cells, NFκB and PARP are induced as a resistance mechanism against gemcitabine treatment that can be inhibited by expression of the adenoviral E1A protein resulting in sensitization of the cells to drug-induced apoptosis [93]. More recently, replication competent wildtype and mutant adenoviruses lacking the anti-apoptotic E1B19K-gene showed increased pancreatic cancer cell killing in combination with gemcitabine by enhancing drug-induced apoptosis. Gemcitabine treatment of pancreatic cancer cells inhibited virus replication completely, but the E1A proteins were still expressed, suggesting that sufficient quantities of E1A were generated by the virus to enable the sensitization of cells to the cytotoxic effects of gemcitabine. This also showed that enhancement was not dependent on a productive viral replication. In addition, the induction was more potent with the adenoviral mutants lacking the anti-apoptotic E1B19K gene. This synergism was also evident in a pancreatic cancer

xenograft model [96]. Ad5/3-delta24 is an adenovirus that utilizes the adenovirus type 3 (Ad3) receptor for entry and that selectively replicates in cancer cells with a deficient retinoblastoma (Rb)/p16 pathway. Given these characteristics, it has been proposed as an OV for ovarian cancer. When Ad5/3-delta24 was used in combination with gemcitabine in ovarian cancer cells, synergistic interactions were observed that resulted in enhanced cell killing. It was also noted that gemcitabine reduced the initial rate of Ad5/3-Delta24 replication but did not affect the total amount of virus produced. In a murine model of peritoneally disseminated ovarian cancer, the administration of Ad5/3-delta24 plus gemcitabine improved the survival of mice, but this enhancement was dependent on the timing and sequencing of the drug and virus [97].

Reoviruses have also been tested in combination with gemcitabine. ReoT3D was tested in combination with gemcitabine and other chemotherapy agents for the treatment of NSCLC cells. It was noted that, unlike other chemotherapy agents tested in this report (*e.g.*, paclitaxel and vinblastine), treatment of NSCLC cells with gemcitabine did not increased virus progeny production, but did result in a modest enhancement of PARP cleavage. However, the synergistic effects were observed only in cells that were sensitive to gemcitabine and not in gemcitabine resistant NSCLC cell lines [78].

Combination therapy using the oncolytic parvovirus H-1PV and gemcitabine has also led to synergistic killing of pancreatic cancer cells and improved overall anticancer effects. Gemcitabine resistant cell lines were shown to be susceptible to H-1PV infection and oncolysis, suggesting the use of H-1PV as a second-line treatment for pancreatic cancers that do not respond to gemcitabine chemotherapy. Thus, H-1PV may improve the therapeutic effect of gemcitabine by enhanced cell killing of drug-sensitive cells and by the eradication of chemotherapy resistant tumors emerging at later stages of drug treatment. In an orthotopic model of pancreatic cancer, H-1PV was effective against tumors that escaped gemcitabine treatment and its subsequent administration after gemcitabine treatment increased survival time. However, when combination therapy was applied simultaneously, H-1PV failed to improve the therapeutic effect of gemcitabine potentially due to negative effects on virus replication. Therefore, a two-step protocol was recommended for combination therapy using H-1PV and gemcitabine [98].

The ability of oncolytic HSVs to synergize with gemcitabine has been shown to depend on the genetic background of the OVs used. Two oncolytic HSV-1 strains, R3616 and hrR3, were tested and compared in combination with gemcitabine for the treatment of pancreatic cancer. Both R3616 and hrR3 are genetically engineered HSVs whose replication has been restricted in normal cells but not in cancer cells through the deletion of genes encoding the ICP34.5 protein or the ICP6 proteins (including the viral RR), respectively. For both viruses, cytotoxicity *in vitro* was enhanced with gemcitabine treatment, but R3616 was more susceptible to this enhancement. The replication of both viruses was inhibited by gemcitabine, but hrR3 was more sensitive to the inhibitory effects of the drug. In a mouse model of pancreatic cancer with peritoneal dissemination, R3616 in combination with gemcitabine showed a greater anti-cancer effect than virus alone (although this difference was not statistically significant) but hrR3 in combination with gemcitabine showed antagonistic effects compared to hrR3 alone. Thus, other oncolytic HSV strains with ICP6

deletions such as G207 may not be compatible with gemcitabine chemotherapy potentially due to the lack of expression of the viral RR [99]. Synergistic interactions between the oncolytic HSV-1 NV1066 strain and gemcitabine have also been reported. NV1066 has been rendered safe via deletions in the viral genes ICP0, ICP4, and $\gamma_1$34.5. Unlike the previous HSV strains, the *in vitro* replication of NV1066 was increased by gemcitabine resulting in an enhancement of cytotoxicity in human pancreatic cancer cell lines [100]. Together these reports suggest that the overall anticancer effects of a drug and OV combination therapy are sometimes dependent on the genetic background of each particular OV.

3.1.2.1. 5-Fluorouracil (5-FU): 5-FU is a pyrimidine antimetabolite, and depending on the derivatives, the toxic effect of this chemotherapy drug can be DNA or RNA directed [101]. Combination therapy with 5-FU and adenoviruses has been explored. A conditionally replicating adenovirus synergized with 5-FU pretreatment in pancreatic cancer cell lines that also translated into a therapeutic benefit *in vivo*. The synergism between 5-FU and this adenovirus vector appeared to be partly due to the upregulation of the Coxsackievirus-adenovirus receptor (CAR) for viral entry by 5-FU [102]. It has also been reported that an increase in virus uptake is associated with 5-FU treatment even in cells with low CAR expression [103]. Combination treatments involving 5-FU and oncolytic adenoviruses has been explored through three major approaches: 1) improving the efficiency of 5-FU prodrug conversion in infected cancer cells with enzymes that metabolize the drug by gene delivery [104-108], 2) sensitizing infected cancer cells to drug with viral delivery of proapoptotic molecules (*e.g.*, wild-type p53) [109-113], and 3) viral delivery of other therapeutic molecules that may enhance the effects of 5-FU [114-116]. Overall the synergism between 5-FU and genetically engineered adenovirus will allow the use of lower drug doses [117] and may help overcome the resistance of particular cancer types to drug treatment. Clinical trials using this combination have been carried out in different cancer types [57, 102, 118].

In addition to adenoviruses, herpesviruses have also shown promising synergistic interactions with 5-FU. The oncolytic HSV-1 NV1066 has been reported to synergize with 5-FU in pancreatic cancer cells by enhancing viral replication. These synergistic effects allowed for a dose reduction for both virus and drug without compromising cytotoxicity [100]. Other oncolytic HSV-1 strains have also shown enhanced cytotoxicity in combination with 5-FU [88, 119]. The HSV-1 oncolytic strain G207 has been shown to synergize with 5-FU resulting in a prolonged survival of mice with peritoneal dissemination of gallbladder cancer. In this report the authors showed that 5-FU treatment increased the intratumoral RR activity and the viral spread within the tumors and suggested this as a potential mechanism of synergy [120].

3.1.3. DNA Intercalating Agents and OVs

3.1.3.1. Doxorubicin: Doxorubicin is an anthracycline that inhibits DNA synthesis by forming a complex between topoisomerase I and II and DNA. By inhibiting the activity of topoisomerase II doxorubicin causes breaks in the genomic DNA [121] and may also increase the R2 subunit of RR [122]. It has been reported that in the presence of wild-type p53, the oncolytic adenovirus ONYX-015 can reverse doxorubicin resistance [123] and also synergize with cisplatin, while drug resistance did not affect and, even improved cancer cell

sensitivity to ONYX-015 [124]. These led to the phase I-II clinical trial of ONYX-015 in combination with mitomycin C, doxorubicin, and cisplatin which showed partial response in advanced sarcoma patients [125, 126]. Additive effects can be achieved by combining of doxorubicin with HSV-1716 (a replication selective HSV-1 lacking the ICP34.5 gene) in a lung cancer model [86] and also with HSV G207 or NV1023 (both lacking ICP34.5 and RR) in anaplastic thyroid cancer (ATC) models [127]. Combination treatments using VSV and doxorubicin have also been reported to induce synergistic interactions. The molecular mechanism behind this synergism was attributed to the VSV-induced degradation of the anti-apoptotic myeloid cell leukemia 1 (Mcl-1) protein [128].

3.1.4. Mitotic Inhibitors

3.1.4.1. Taxanes (Paclitaxel and Docetaxel): Paclitaxel and Docetaxel are anti-microtubule agents that promote the assembly of microtubules from tubulin dimers and stabilize microtubules preventing their depolymerization. This stability results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital mitotic and non-mitotic cellular functions [129, 130].

Paclitaxel has been reported to synergize with the oncolytic HSV G207 in killing ATC cells, without improving G207 replication *in vitro*. Cells treated with combination therapy had significantly higher acetylation of a-tubulin and mitotic arrest, which lead to enhanced apoptosis when compared to either treatment alone. This enhancement *in vitro* also translated into a therapeutic benefit *in vivo* [127]. One of the mechanisms by which ATC cells gain resistance to paclitaxel is by the upregulation of the Raf/MEK/ERK pathway that in turn can enable the efficient replication of ICP34.5 deleted oncolytic HSVs [131]. Thus, in this case, oncolytic virotherapy complements conventional chemotherapy by targeting drug resistant cancer cells. Increased intratumoral spread of oncolytic HSV-1 has also been reported upon pretreatment of mammary tumors with paclitaxel or apoptosis inducers such as the TNF-related apoptosis-inducing ligand (TRAIL) or CD8/caspase 8 [132]. The attenuated oncolytic HSV-1 HF10, has also shown synergistic interactions with paclitaxel that resulted in prolonged survival in a murine peritoneal dissemination model of colon cancer when compared with single treatments [133] and suggests a broad application of this OV-drug combination in other advanced cancer types. In a prostate cancer model, the engineered HSV-1, G47Delta, combined with taxanes (docetaxel and paclitaxel) also showed augmented oncolysis *in vitro* and *in vivo*. In particular, when G47Delta was used in combination with docetaxel the virus dose could be reduced 10-fold without compromising the oncolytic effect. Mechanistically, G47Delta enhanced the apoptosis of taxane-treated, mitotically arrested cells by increasing their premature exit from mitosis [134]. Given these synergistic interactions of oncolytic HSVs with taxanes, a specific gene-directed enzyme prodrug therapy (GDEPT) system was designed in which a paclitaxel prodrug was used in combination with an HSV amplicon expressing a prodrug converting enzyme and HSV-1 HF10 as a helper virus. In this system, the prodrug converting enzyme would be expressed by the amplicon at high levels in cancer cells resulting in enhanced paclitaxel activation within the tumors. Increase activation of paclitaxel in the tumors would in turn enhance the replication of the oncolytic HSV HF10. Increased cytotoxicity was reported using this system but only in cells with low susceptibility to HF10 [133]. Therefore, this combined

therapy provides a strategy for targeting malignancies with low sensitivity to an oncolytic HSV by enhancing the activation of a drug that in turn increases the efficacy of an OV specifically in tumor cells.

In an early study, it was suggested that adenovirus-based p53 gene therapy led to synergistic interactions with paclitaxel in various cancer models, including human head and neck, prostate, ovarian, and breast cancer [135]. A prostate cancer-specific adenovirus, CV787, was shown to synergize with paclitaxel and docetaxel *in vitro* and *in vivo*. The mechanism of the observed synergistic effects *in vivo* was reported to be via elevated viral yields within the tumor mass upon taxane administration and dependent on the upregulation of p53 potentially sensitizing the cells to apoptosis [136]. A chimeric adenovirus, Ad5/35, containing the adenovirus subgroup B (Ad35) fiber armed with the human TRAIL transgene was able to infect cancer cells refractory to adenovirus subgroup C (Ad5) oncolysis and synergized with paclitaxel [137]. In a study utilizing this virus, synergism between docetaxel and CG8840 led to regression of human bladder cancer xenografts [138]. In a pancreatic cancer model, the oncolytic potential of adenovirus was shown to be improved by genetic alterations in its genome, and its efficacy was further enhanced by combination with paclitaxel, although complete tumor regressions were not achieved [139]. In another study, pre-treatment of cancer cells with paclitaxel or cisplatin restored the cytotoxicity of attenuated E1A expressing E3B mutant adenoviruses to levels comparable with wildtype Ad5 in several but not all cancer cell lines tested. In the presence of drug, virus uptake and E1A expression were enhanced. However, this drug induced enhancement did not occur with viruses lacking the E1A protein [85]. In fact, the expression of the adenovirus E1A protein has been shown to sensitize cancer cells to paclitaxel-induced apoptosis [140] and to lysis by macrophages [141]. In animal models, enhancement was more dramatic in immunocompetent animal models [85], suggesting that the synergistic effects were due to the effects of the adenovirus E1A protein on the cancer cells and on the immune response of the host [140, 141].

In addition to oncolytic adenoviruses, paclitaxel can also potentiate the oncolytic activity of ReoT3D in human NSCLC cells by increasing mitotic arrest and apoptosis [78]. Furthermore, the synergistic effect of paclitaxel with ReoT3D that resulted in enhanced cytotoxicity did not appear to be related to the initial sensitivity of the cells to either agent alone. Importantly, ReoT3D showed superior synergistic capacity with paclitaxel than with vinblastine and gemicitabine [78]. Thus, in choosing appropriate combination treatments, factors including cancer type, OV type, and the mode of action of the drug need to be considered.

3.2. Non Cytotoxic Targeted Chemotherapy Agents

Many classes of targeted agents with anti-tumor activity have been developed that range from tyrosine kinases and small molecule inhibitors to monoclonal antibodies. Importantly, some of these drugs have also been shown to have synergistic interactions with a wide range of OVs. The mechanisms of synergy are only starting to be elucidated. Some of these OV-drug combinations promote synergy at the level of the organism and its immune response while others work on intracellular pathways. The use of these drugs to enhance OV therapy

proves to be an attractive idea. However, care must be taken to ensure that the signaling pathways targeted by these drugs are not factors required for an OV's selective and efficient replication in cancer cells.

3.2.1. Histone Deacetylase Inhibitors (HDIs)—HDIs such as valproic acid (VPA), Suberoylanilide hydroxamic acid (SAHA) and Trichostatin A (TSA) have gained interest in the OV field due to their ability to suppress the transcriptional activation of IFN stimulated genes (ISGs). HDIs inhibit the activation of ISGs in response to IFN treatment or via IFN regulatory factor (IRF)-3 induction upon virus infection [142]. Several reports have shown that combination treatments involving OVs and HDIs result in an overall enhancement of the anticancer effect and that these enhancement is primarily due to the inhibitory effects that HDIs have on the innate immune responses of the infected tumor cell. This is in contrast to CPA's effects, which are not exerted directly upon the infected tumor cell, but rather on the cells of the host's immune system.

Clinically approved HDIs such as SAHA (Voronistat) can potentiate the oncolytic effects of VSV. SAHA treatment increased the infectivity and replication of VSV concomitant with an increase in apoptosis of cancer cells both in cell culture and *in vivo* [143]. In addition, these effects correlated with the inhibition of the IFN response as measured by a reduction in the expression levels of IFNβ, MxA and IRF-7 [143]. Also, HDI treatment rendered tumors that are normally resistant to VSV susceptible to VSV oncolysis. Resistance to VSV was restored and rapid clearance of virus from the tumors was observed upon withdrawal of the HDI [143]. Thus, HDIs can be used as a switch to control virus replication in these tumors. Importantly, HDI treatment did not alter the tumor specificity of VSV and normal tissues were not infected in animals receiving the combination treatment. Other viruses, such as oncolytic VACV also showed enhanced spread and replication with HDIs. [143]. Several HSV-1 based OVs also showed enhanced replication and cytotoxicity *in vitro* and *in vivo* due to impaired IFN responses [144].

It has been well documented that HDIs also target other proteins besides histones. The pleiotropic effects of HDIs on cellular signaling pathways have been exploited to create synergism with OVs. In particular, HDIs have been shown to affect the function of transcription factors such as p53 and NFκB, cell cycle regulators such as the cell cycle kinase inhibitor p21 and apoptosis regulators as reviewed in [145, 146]. Thus, HDI treatment may alter the replication of OVs that rely on any of these molecules during their life cycle. For instance, early during HSV-1 infection, activation of NFκB is required for progression of the virus replication cycle. In infected cells, HSV-1 utilizes NFκB for the transcription of early gene products [147]. For the oncolytic HSV-1 R849, treatment of cells with TSA resulted in an increase in viral titers and cytotoxicity due to drug-induced upregulation of acetylated p65 [148].

HDI treatment of cancer cells may also promote an increase the expression of CAR, enhancing the uptake of both replication competent and incompetent adenoviruses [149, 150]. The upregulation of CAR appears to be the result of the acetylation of its promoter region upon treatment with HDIs [151]. In addition, it has been noted that the upregulation of CAR occurs primarily in cancer cells and not in normal cells [149]. The replication and

the oncolytic effects of the adenoviruses OBP-301 and dl520 were enhanced by combination treatments with the HDIs FR901228 and TSA, respectively [152, 153]. Contrary to these findings, it has been reported that VPA inhibits adenovirus replication at a late stage during the virus replication cycle through VPA's induction of p21 [154]. Thus, the effects of HDIs on the replication of competent adenoviruses remain unclear and need to be further evaluated taking into consideration differences in OV strains and in the potential differences between HDIs.

Another way in which HDIs may synergize with adenovirus based OV therapy is by specifically upregulating the expression levels of transgenes widely used in adenovirus-based gene therapy, thus enhancing the overall therapeutic effects of these viral vectors. In most cases, the enhanced transgenes are themselves a target of HDIs or they interact with proteins whose function is influenced by HDIs. Specifically, HDIs have shown to enhance p53 and TRAIL based gene therapies. The adenovirus-delivered wildtype p53 protein has been shown to be hyperacetylated upon HDI treatment which correlated with an enhancement of p53 mediated apoptosis. Several *in vivo* models have shown a significant increase in the effectiveness of the p53 adenovirus based gene therapy when used in combination with HDIs [155-158]. Adenovirus-based TRAIL gene therapy has also been shown to be enhanced when combined with HDIs. Combination therapy usually resulted in an enhancement of apoptosis in cancer cells tested. The mechanism of this enhancement seems to be cell type specific [159-162].

3.2.2. Rapamycin—There are several known mechanisms responsible for the synergism observed between rapamycin and OV therapy. Rapamycin specifically inhibits the mammalian target of rapamycin complex 1 (mTORC1). Importantly it has been reported that rapamycin-induced inhibition of mTORC1 and its downstream mediators results in impaired type I IFN production [163]. Recently, rapamycin was reported to synergize with VSV in an immunocompetent model of malignant glioma, by inhibiting type I IFN production after VSV infection. Impaired IFN production in the infected cancer cells led to an increase in VSV replication and subsequently in the anticancer effect both *in vitro* and *in vivo* [164]. Aside from its effect on cellular innate immune responses, rapamycin also has an inhibitory effect on cells of the immune system *in vivo*. Rapamycin's negative effects on protein synthesis and cell-cycle progression lead to the inhibition of T and B cell proliferation, differentiation and antibody production [165]. Rapamycin has also been shown to synergize with oncolytic poxviruses such as the attenuated VACV strain vvDD [65].

Combination therapy using rapamycin and MYXV, a rabbit specific oncolytic poxvirus, enhances replication of MYXV *in vitro* [166, 167] and its oncolytic effects in several cancer models [168-170]. One of the factors that contribute to the tropism of MYXV for cancer cells are the levels of activated Akt [37]. MYXV encodes a protein called M-T5, which directly interacts with Akt and the absence of M-T5 restricts the replication of MYXV to cancer cell lines with high levels of constitutively active Akt [37, 171, 172]. Akt is a serine/threonine kinase that is phophorylated by mTORC2 and is involved in many cellular signaling pathways and its upregulation usually contributes to tumorigenesis [173, 174]. The susceptibility to MYXV infection in certain cancer cell lines with moderate levels of Akt can be enhanced by pretreatment with rapamycin. In these cells, rapamycin treatment caused

an increase in the levels of phophorylated Akt which correlated with enhanced MYXV replication and spread [166].

Replication competent adenoviruses were the first OVs reported to synergize with rapamycin [175, 176]. Rapamycin has been reported to induce autophagy [177] and several reports have described enhancement of autophagy when adenoviruses were used in combination with rapamycin. The oncolytic adenovirus OBP-405 has been shown to induce autophagy as a mechanism of tumor cell killing [178] and combination treatment with rapamycin resulted in enhanced anticancer effect in glioma models presumably through convergence of both treatments into the autophagic pathway [178]. Another oncolytic adenovirus Delta-24-RGD also synergized with rapamycin through the autophagic pathway and upregulation of Atg5 in combination treated tumors was reported[179].

3.2.3. Cyclooxygenase 2 (COX-2) Inhibitors—COX-2 inhibitors are members of a nonsteroidal class of anti-inflammatory drugs. The upregulation of COX-2 promotes tumorigenesis by enhancing the levels of its direct substrate (prostaglandin E2) and by promoting angiogenesis through enhanced expression of VEGF among other effects as reviewed in [180]. Reports have shown that COX-2 is expressed in B lymphocytes and that the use of COX-2 inhibitors decreases antibody production [181]. Recently, combination therapy with the COX-2 inhibitor Celecoxib and oncolytic VACV showed synergistic anticancer effects in an ovarian cancer model [53]. The immunosuppressive effects of the COX-2 inhibitor decreased the production of neutralizing antibodies against VACV and allowed for a second dose of virus to be administered. The infiltration of macrophages and CD8+ T cells was not affected, although a slight reduction in CD4+ T cells was observed [53]. Treatment of ovarian cancer cells with the COX-2 inhibitor did not result in an enhancement of virus replication *in vitro*, therefore its synergistic effects in combination with VACV are probably due to its immunosuppressive effects *in vivo* [53].

3.2.4. Epidermal Growth Factor Receptor (EGFR) Inhibitors—Erlotinib is a small molecule inhibitor that targets the EGFR signaling pathway. Erlotinib binds to the ATP binding site of EGFR and inhibits its autophosphorylation and downstream signal transduction events. Erlotinib has anti-proliferative effects in cancers with upregulated EGFR signaling and has been approved for use in combination with gemcitabine for the treatment of pancreatic cancer [182]. Erlotinib has been tested in combination with oncolytic HSVs. Two oncolytic HSV viruses G307 and hrR3 were tested in combination with erlotinib in a malignant peripheral nerve sheath tumor model exhibiting aberrant EGFR signaling. *In vitro* combination therapy utilizing HSV and erlotinib showed an additive effect suggesting that inhibition of the EGFR receptor signaling pathway did not affect the replication of the viruses. In subsequent animal models, combination treatment resulted in a modest increase in the overall anticancer effect, but further evaluation of this combination was proposed [183].

3.3. Arming Oncolytic Viruses to Enhance chemotherapy

The use of second generation armed OVs that express therapeutic transgenes offers a multitude of possibilities for the enhancement of virotherapy as a monotherapy [184, 185].

However, OVs can also be specifically armed with trangenes that can enhance drug-based therapies by chemosensitizing the infected cancer cell to the effects of a particular drug. Most of these systems are based on gene-delivery enzyme prodrug therapy (GEDPT), in which viruses are used to selectively deliver enzymes to cancer cells that will convert prodrugs into their active, toxic metabolites. These approaches aim at increasing the selectivity of chemotherapy and thereby reducing systemic toxicity commonly associated with these treatments. Importantly, some of these systems are also associated with an efficient bystander effect that also contributes to the overall enhancement of the anticancer effects. However, for these systems to be effective, the drug-converting enzyme must be selectively and efficiently expressed in tumors.

3.3.1. Cytidine Deaminase/Uracil Phosphoribosyltransferase/5-FU System—

OVs aimed at enhancing 5-FU based chemotherapy are armed with enzymes such as cytosine deaminase (CD) or uracil phosphoribosyltransferase (UPRT) that enhance the intracellular processing of 5-FU prodrugs. Cytidine deaminase is a pyrimidine salvage enzyme that can be derived from bacteria (*Escherichia coli*) or yeast (*Saccharomyces cerevisiae*) and that converts the prodrug 5-fluorocytosine (5-FC) into the cytotoxic agent 5-FU. In turn, UPRT converts 5-FU into its main toxic metabolite, 5-fluoro-2'-deoxyuridine monophosphate (5-fluoro-dUMP) [186], which inhibits the cellular thymidylate synthase enzyme and subsequently DNA synthesis [187]. Oncolytic poxviruses, herpesviruses, VSV, and adenoviruses expressing one or both of these enzymes have been engineered for combination with 5-FU based chemotherapy.

Oncolytic VACVs have been armed with CD (VV-CD) [188, 189] or with a CD/UPRT fusion transgene (VV-FCU1) [190] for use in combination with 5-FC. *In vitro*, both viruses showed a reduction in viral progeny production when used in combination with 5-FC. However, in animal models combination therapy was more effective than either treatment alone [188, 190]. VV-CD has been tested in models of colon and ovarian cancer [188, 189], while VV-FCU1 was tested in models of metastatic colon cancer [190].

The oncolytic HSV-1 M012 expressing the CD trangene under the control of the cellular early growth response promoter 1 (Egr-1) was constructed for use in combination with 5-FC for the treatment of malignant brain tumors. Even though the replication of M012 was inhibited by the addition of 5-FC *in vitro*, cells that were treated with virus and drug showed enhanced cytotoxicity compared to drug or virus alone. Enhanced bystander killing of HSV-1 resistant cells was also reported. In animal models, tumor growth was reduced significantly in groups treated with M012 and 5-FC when compared to groups treated with the parental (unarmed) virus and drug [191]. Another oncolytic HSV-1 armed with CD, OncoVEX(GALV/CD), showed enhanced cytotoxic effects *in vitro* when used in combination with 5-FC in head and neck squamous carcinoma cell lines that were less susceptible to the oncolytic effects of the virus [192]. An armed VSV expressing a CD/UPRT fusion gene combined with 5-FC also reported enhancement of cell killing and a considerable bystander effect *in vitro*. *In vivo*, this armed VSV significantly inhibited tumor growth in a syngeneic lymphoma model and in a mammary carcinoma model [193].

A replication competent adenovirus, Delta24, expressing a “humanized” version of the yeast CD transgene was constructed for use in combination with 5-FC for the treatment of malignant gliomas. This armed adenovirus showed improved cytotoxicity *in vitro* along with a bystander killing effect. In animal models, the armed virus proved more effective in combination with 5-FC [194]. Another report utilizing a Wnt-targeted replication competent adenovirus in which the yeast CD transgene was expressed using an internal ribosomal entry site (IRES) showed that the armed adnenovirus in combination with 5-FU was also a more efficacious therapy compared to single therapies in human colon cancer cells *in vitro*. In this same report, the greatest enhancement was seen in colon cancer cell lines that were more resistant to infection with the parental oncolytic adenovirus [195].

Other armed viruses expressing other therapeutic genes such as second mitochondria derived activator of caspases (Smac) [196], manganese superoxide dismutase (MnSOD) [197] and even viral fusion proteins by themselves or along with the CD transgene [192, 198], have also been tested in combination with 5-FU chemotherapy and shown to be effective. Combination of two GEPT systems has also been reported. A conditionally replicating armed adenovirus, AxE1CAUT, expressing the UPRT and the HSV thymidine kinase (TK) genes was engineered to work in combination with both 5-FC and ganciclovir and proved to be advantageous for the treatment of human bile duct cancer [104].

3.3.2. CB1954 and Nitroreductase (NTR)—CB1954 is a poorly metabolized monofunctional DNA alkylating agent with low toxicity, which is converted into a functional cytotoxic alkylating agent by *E. Coli* nitroreductase (NTR) [199]. Since the drug is highly membrane permeable and readily diffuses, it potentiates an efficient bystander effect [200, 201]. Combination treatment with an oncolytic HSV-1 expressing NTR, HSV1790, and CB1954 resulted in enhanced tumor cell killing *in vitro*, reduced the tumor burden and improved survival *in vivo* compared to either therapy alone [202]. A replication competent adenovirus (dl1520) expressing NTR has also shown similar effects in colon cancer cells [203].

3.3.3. CYP2B1 *Gene and CPA*—The rat CYP2B1 gene encodes for a cytochrome P450 enzyme that can convert cyclophosphamide (CPA) into its active metabolites [204]. The oncolytic HSV-1 rRp450 expressing the CYP2B1 was engineered for use in combination with CPA. Oncolytic rRp450 showed increased cytotoxicity *in vitro* and was able to safely decrease tumor burden in a diffuse liver metastases model when used in combination with CPA [205, 206]. In another report, a two GEPT system using an oncolytic HSV-1 armed with both CYP2B1 and carboxylesterase was generated for use in combination with CPA and irinotecan (a topoisomerase 1 inhibitor) chemotherapies [207]. Carboxylesterase converts irinotecan into its toxic metabolite SN-38 [208]. The use of this armed virus in combination with CPA and irinotecan enhanced the oncolysis of glioma cells *in vitro* and in animal models. Importantly, the combination of these two drugs did not affect the replication of this OV [207].

3.3.4. Fludarabine and Nucleoside Phosphorylase (PNP)—Fludarabine is a purine analog commonly used as a chemotherapy for hematological malignancies [209, 210]. The *E. coli* PNP converts purine analogs into highly diffusible and toxic metabolites capable of

incorporating into RNA and DNA and of producing a potent bystander effect [211, 212] . A CD20 targeted and PNP armed measles OV was generated for use in combination with fludarabine for the treatment of mantle cell lymphoma [213]. *In vitro*, administration of the drug early during virus replication controlled virus spread at later time points during infection and enhanced cell killing. Enhanced oncolytic effects and longer survival times were observed using combination treatment in a Burkitt's lymphoma xenograft model [213]. Similarly a carcinoembrionic antigen (CEA) - targeted and PNP armed measles virus (MV-PNP-antiCEA) was constructed for its use in a syngeneic colon cancer model. In this model, the armed OV was combined with the prodrug, 6-methylpurine-2'-deoxyriboside (MeP-dR), another substrate for PNP [211]. Combining this prodrug with MV-PNP-antiCEA enhanced the oncolytic effects *in vivo*. In addition, the use of CPA in this immunocompetent model retarded the production of MV neutralizing antibodies and further enhanced the oncolytic effects. With this triple combination treatment (prodrug, armed MV and immunosuppressant) 9 out of 10 animals showed complete remission [69].

4. SUMMARY AND FUTURE DIRECTIONS

OVs are unique agents with the potential to selectively eliminate cancerous cells while sparing normal cells and tissues. The inherent ability of OVs to target tumor cells has led to their potential applications as cancer treatment alternatives. Importantly, the unique mechanisms of action for each OV vary greatly from the mechanisms of action of existing cancer chemotherapeutics. Thus, the distinct mechanisms of oncolysis between chemotherapy agents and OVs, may make these therapies complementary and their selective combination may result in a more efficient way to improve treatment outcome for certain cancer patients. Numerous studies have shown that many OVs can synergize with particular chemotherapy drugs in preclinical animal models. There are several potential advantages of creating synergy with chemotherapy and virotherapy. In the case where chemotherapy drugs improve the efficacy of OVs, administration of a lower dose of OV may be achieved thereby reducing any potential complications of OVs without compromising the overall efficacy. On the other hand, if the OV used improves the efficacy of chemotherapy, a lower dose or shorter period of drug treatment may be achieved, consequently reducing any side effects or drug toxicity and also reducing the possibility of acquiring resistance to the drug.

However, in order for patients to obtain the maximum benefits from these combination treatments, a better understanding of the mechanism(s) that lead to synergistic interactions between chemotherapy drugs and OVs are required, including also a more complete understanding of the mechanisms of tumor cell killing that occur during drug and OV monotherapies. Mechanistically, it is possible that the synergistic interactions observed between certain drugs and OVs are due primarily to the enhancement of oncolytic pathways observed in the individual therapies or, alternatively, due to the activation of novel pathways that are only triggered during the combination treatment. In addition, the understanding of the limitations of drug and OV therapies is critical for the improvement of the individual therapies. In particular for OVs, it is clear that more efficient delivery methods to tumor sites are required as well as more enhanced spread of the virus within the tumors, while for chemotherapy agents, major limitations include multidrug resistance, high toxicities or poor efficacy (in the case of some targeted agents). Improvement upon the individual therapies

will also bring about improvements on drug-OV combination therapies that can ultimately translate into enhanced therapeutic benefits and more efficiently tailored cancer treatments for patients. Finally, a successful treatment outcome involving drug-OV combinations may be possible through the careful selection of the chemotherapy agent(s) and the OVs, taking also into consideration the cancer type and the mechanism(s) of action for the combined treatments.

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Table 1

Examples of Replication Competent Oncolytic Viruses

Genome Type	Family	Examples	Reference
dsDNA	Adenoviridae	Human Adenoviruses serotype 5 (Ad5)	[18, 19]
	Herpesviridae	Herpes simplex type 1 (HSV-1)	[20]
	Poxviridae	Vaccinia virus (VACV) Myxoma virus (MYXV)	[21, 22]
ssDNA	Parvoviridae	Parvovirus H1	[23]
(+) ssRNA	Coronaviridae	Murine hepatitis virus (MHV)	[24]
	Picornaviridae	Poliovirus PVS-RIPO	[25]
	Retroviridae	Murine leukemia virus (MLV)	[26, 27]
	Togaviridae	Sindbis virus	[28]
(−) ssRNA	Orthomyxoviridae	Influenza A virus	[29]
	Paramyxoviridae	Measles virus (MV) New castle disease virus (NDV)	[30, 31]
	Rhabdoviridae	Vesicular stomatitis virus (VSV)	[32]
dsRNA	Reoviridae	Reovirus type 3 Dearing (T3D)	[33]

Table 2

Examples of Chemotherapy and OV Combination Treatments with Known Mechanisms of Synergy

Chemotherapy Agent	OVs	Primary Mechanism of Synergy with OVs
Cyclophosphamide	Herpesviruses Reovirus Adenoviruses Poxviruses Paramyxoviruses	Suppression of the host's innate and adaptive anti-viral immune responses leads to increased and prolonged viral replication
Cisplatin	Herpesviruses with $\gamma_1$34.5 deletions	Upregulation of GADD34 improves viral replication
Gemcitabine	Adenoviruses	E1A expression counteracts drug resistance mechanisms
5-FU	Adenoviruses Herpesviruses	Upregulation of CAR Upregulation of cellular RR
Doxorubicin	VSV	Viral degradation of Mcl-1 enhances drug sensitivity
Taxenes	Herpesviruses with $\gamma_1$34.5 deletions Adenoviruses	Virus replication is enhanced in drug resistant cells with an upregulated Raf/MEK/ERK pathway E1A expression increases drug sensitivity and anti-tumor immune responses
Histone deacetylase inhibitors	Rhabdoviruses Herpesviruses Poxviruses Adenoviruses	Inhibition of IFN production in infected cancer cells leads to enhanced viral replication Upregulation of CAR and Upregulation of virally delivered transgenes
Rapamycin	Rhabdoviruses Poxviruses Poxviruses (MYXV) Adenoviruses	Impaired IFN production in infected cancer cells enhances viral replication Upregulation of phosphorylated Akt Enhancement of autophagy
Cyclooxygenase 2	Poxviruses	Impaired anti-viral antibody production leading to increased viral replication