Lymphoma Chemovirotherapy: CD20-Targeted and Convertase-Armed Measles Virus Can Synergize with Fludarabine

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Abstract
Combination chemotherapy regimen incorporating CD20 antibodies are commonly used in the treatment of CD20-positive non-Hodgkin's lymphoma (NHL). Fludarabine phosphate (F-araAMP), cyclophosphamide, and CD20 antibodies (Rituximab) constitute the FCR regimen for treating selected NHL, including aggressive mantle cell lymphoma (MCL). As an alternative to the CD20 antibody, we generated a CD20-targeted measles virus (MV)--based vector. This vector was also armed with the prodrug convertase purine nucleoside phosphorylase (PNP) that locally converts the active metabolite of F-araAMP to a highly diffusible substance capable of efficiently killing bystander cells. We showed in infected cells that early prodrug administration controls vector spread, whereas late administration enhances cell killing. Control of spread by early prodrug administration was also shown in an animal model: F-araAMP protected genetically modified mice susceptible to MV infection from a potentially lethal intracerebral challenge. Enhanced oncolytic potency after extensive infection was shown in a Burkitt's lymphoma xenograft model (Raji cells): After systemic vector inoculation, prodrug administration enhanced the therapeutic effect synergistically.

In a MCL xenograft model (Granta 519 cells), intratumoral (i.t.) vector administration alone had high oncolytic efficacy: All mice experienced complete but temporary tumor regression, and survival was two to four times longer than that of untreated mice. Cells from MCL patients were shown to be sensitive to infection. Thus, synergy of F-araAMP with a PNP-armed and CD20-targeted MV was shown in one lymphoma therapy model after systemic vector inoculation. [Cancer Res 2007;67(22):10939–47]

Introduction
Oncolytic virotherapy, the treatment of cancer patients with replication-competent viruses, has progressed from preclinical experiments to phase III clinical trials in humans (1–3). The clinical trials have shown the safety of this approach, and oncolytic viruses are now combined with chemotherapy (4) and radiotherapy (5), or suicide gene therapy (6), to increase efficacy. The first oncolytic therapy regimen was recently approved (7).

The oncolytic efficacy of recombinant measles viruses (MV) is being assessed in an ovarian cancer trial nearing completion and in recently opened glioma and myeloma clinical trials. A phase I study of i.t. injections of the unmodified MV Zagreb vaccine strain in patients with cutaneous T-cell lymphomas showed safety as well as clinical responses (8).

MV lymphotropism is an asset for lymphoma treatment. Spontaneous tumor regressions of lymphoma (9) and leukemia (10) was repeatedly observed after contagion with wild-type MV. Experimental studies based on human tumor xenografts in immunodeficient mice treated with a MV vaccine lineage strain confirmed a strong oncolytic effect (11, 12).

MV oncolytic specificity can be retargeted based on single-chain antibodies displayed on the viral attachment protein, hemagglutinin (H; refs. 13, 14). For hematologic malignancies, viruses recognizing CD20 (15) and CD38 (16) were generated; however, lack of information about the mechanism of MV cell entry through the natural receptors SLAM (17) and CD46 (18) initially did not allow the production of recombinant viruses exclusively recognizing the target protein. The recent identification of the H protein residues essential to enter cells through the natural receptors (19) allows generation of viruses fully retargeted at the cell entry level (20, 21).

Non-Hodgkin's lymphoma (NHL) is the most common hematologic malignancy and accounts yearly for ~18,000 deaths in the United States (22). Therapeutic options include chemotherapy, radiotherapy, and biological agents, including antibodies against CD20 (23). The FCR therapeutic regimen, a combination of fludarabine phosphate (F-araAMP), cyclophosphamide, and the Rituximab CD20 monoclonal antibody, is a frontline treatment for selected NHL, and is used as a salvage regimen in mantle cell lymphoma (MCL; refs. 24, 25).

The integral surface membrane protein CD20 is expressed on both normal and neoplastic B cells but not in other tissues (26). CD20 monoclonal antibodies can be effective therapeutics for NHL (27). Their mechanism of action is not fully understood; however, immune-mediated effects and antibody-dependent cell-mediated cytotoxicity in combination with direct effects of CD20 binding may cause cytotoxicity (28). Levels of B cells are temporarily strongly reduced by anti-CD20 therapy; despite this, adverse events are minimal. Thus, B-cell depletion through a CD20-targeted virus may be well tolerated.

To improve efficacy of the FCR regimen, we seek to substitute the CD20 antibody with a CD20-targeted virus armed with a prodrug convertase locally activating F-araAMP. The active metabolite of F-araAMP, fludarabine, can be activated by the...
prodrug convertase *Escherichia coli* purine nucleoside phosphor-
ylase (PNP) to 2-fluoroadenine. PNP also converts 6-methylpurine-2’-deoxyriboside (MeP-dR) to 6-methylpurine. 2-Fluoroadenine and 6-methylpurine are highly diffusible and can be metabolized to toxic ATP analogues, which inhibit DNA, RNA, and protein synthesis immediately (29). In a conventional approach without PNP, fludarabine is not metabolized to 2-fluoroadenine and, therefore, is not as effective. Thus, local activation of F-araAMP by PNP should enhance its therapeutic efficacy.

In this study, we generated a MV that can enter cells exclusively through CD20, and armed it with PNP. We show in a Burkitt’s lymphoma preclinical model that this oncolytic vector activates fludarabine locally, enhancing its therapeutic effect synergistically after systemic administration. In a MCL model, oncolytic efficacy of i.t. vector administration was very high.

**Materials and Methods**

**Cell culture.** Vero African green monkey kidney cells, HT1080, and human Burkitt’s lymphoma Raji cells were purchased from American Type Culture Collection (ATCC). MCL cells (Granta 519) were a gift of Dr. Thomas Witzig (30). All cells were grown at 37°C in medium recommended by the ATCC in a humidified atmosphere of 5% CO2, Vero-x-αHs, Vero-SLAM, and HT1080-CD20 were generated by stable transfection of the parental Vero and HT1080 cells, respectively, as described previously (15, 21, 31).

**Patient samples.** Primary MCL cells were recovered from surgically removed spleen tissue (two patients) or peripheral blood (one patient). Fresh tissue was cut and minced over a wire mesh to obtain a single-cell suspension. The cell suspension was overlaid on Ficoll-Hypaque (GE Healthcare) and centrifuged to isolate the mononuclear cell layer. Diluted peripheral blood cells were similarly centrifuged on Ficoll. The cells were washed with RPMI 1640 and then aliquoted into 1 mL cryovials in freezing medium (RPMI 1640, 10% DMSO, and 20% FCS), and samples were frozen in liquid nitrogen freezers for long-term storage. For flow cytometry, the cultured cells were run for fluorescence-activated cell sorting (FACS) analysis using a Becton Dickinson FACSCalibur. Acquisition and analysis was done using Becton Dickinson CellQuest Pro software. Ten thousand events were collected both ungated and gated on the live population as determined by scatter properties for each sample.

**Construction of recombinant MV.** The *E. coli* PNP gene was PCR amplified from pSV-PNP (32) using primers providing the appropriate *Mlu*I and *Aat*II restriction sites. The *Mlu*I/Aat*II-digested PCR product was cloned into the MV full-length cDNA using the corresponding restriction sites, resulting in p(+)-JMVPNP. The plasmid pCGHmutCD20 (15) was used as template to insert the CD46- and SLAM-ablating mutations at residue positions 481, 533, 548, and 549. The sequence coding for the H6 peptide was synthesized as single-stranded oligonucleotides in reverse complementary orientation, and subcloned via *Ssp*I and *Spe*I in pCGHmutCD20. The pCGHmutCD20–diagonal fragment was targeted with the retroviral H was exchanged for the corresponding fragment of p(+)-JMVPFP and p(+)-MV-PNP, respectively. The resulting full-length cDNAs were named p(+)-JMVPNPmutHmutCD20 and p(+)-MV-PNPmutHmutCD20. For rescue of fully retrofused viruses, a modified system was used as described previously (21). To prepare virus stocks, Vero-αHs cells were infected with each MV at a multiplicity of infection (MOI) of 0.03 and incubated at 37°C for 36 h. Viruses were harvested by one freeze-thaw cycle from their cellular substrate resuspended in Opti-MEM (Invitrogen). Titers were determined by 50% tissue culture infectious dose (TCID50) titration on Vero-αHs cells.

**Immunoblot analysis.** Viral samples (5,000 TCID50) were directly mixed with an equal volume of SDS loading buffer [130 mmol/L Tris (pH 6.8), 20% glycerol, 10% SDS, 0.02% bromophenol blue, and 100 mmol/L DTT]. These samples were denatured for 5 min at 95°C; fractionated on a 10% SDS-polyacrylamide gel; blotted to polyvinylidene difluoride membranes (Bio-Rad); and immunoblotted with anti-measles nucleocapsid (N) protein antibody (Novus Biologicals) at 1:5,000 dilution, with anti-PNP antibody (a kind gift of Dr. Jeong S. Hong) at 1:10,000 dilution, and with anti-measles H protein antibody at 1:10,000 dilution according to BM Chemiluminescence Blotting kit (Roche). After washing the primary antibody, the secondary horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG antibody (KPL) was applied at 1:10,000 dilution for 3 h at 37°C. The TCID50 was determined by the method of Kärber (33) on Vero-αHs cells. At the end of the incubation period, free viruses were removed and cells were maintained in the appropriate medium. At 36 h after infection, cells were photographed under phase contrast or fluorescence microscopy, respectively.

**Viruses infection and titration.** Each cell line (5 × 10^6 adherent cells or 10^6 suspension cells in a six-well plate) was incubated with each MV at a MOI of 0.5 in Opti-MEM for 3 h at 37°C. The TCID50 was determined by the method of Kärber (33) on Vero-αHs cells. At the end of the incubation period, free viruses were removed and cells were maintained in the appropriate medium. At 36 h after infection, cells were photographed under phase contrast or fluorescence microscopy, respectively.

**Cell viability assay.** Cell viability was determined by 3-(4,5-dimethyl-
diazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Cell Proliferation kit I, Roche) assay. Cells were grown in 96-well microtiter plates (5 × 10^4 per well) in the recommended culture medium in presence or absence of prodrug, respectively. Infection at MOI 0.01, 0.1, 1, and mock was done by adding the corresponding virus preparation to the cells. Cell viability was measured by dye absorbancy as determined by absorbance measurement at 595 nm on an automated ELISA reader. Viability of cells treated with or without prodrugs after infection with different MOIs or uninfected, respectively, was calculated as the mean of quadruplicate absorbance values divided by the mean of quadruplicate absorbance values of ideally cultured cells in the absence of prodrug and virus (control cells) and expressed as percentage of control cells. The MTT assay was done as recommended by the manufacturer.

**Measurement of E. coli PNP activity.** Crude cell extracts were prepared as described previously (34) from s.c. implanted Raji cell tumors (human lymphoma) transduced with a total dose of 5 × 10^6 MV-PNP HmutmutCD20, obtained 6 days after first virus administration. The extracts were incubated with various concentrations of MeP-dR, and the formation of product was measured by high-performance liquid chromatography analysis of the reaction mixture. Activity was expressed as PNP units; one unit represents 1 nmol MeP-dR–converted/mg protein/h.

**In vivo experiments for assessment of safety.** All experimental protocols were approved by the Institutional Animal Care and Use Committee. Transgenic, 8-week-old Ifnar–/–CD46Ge mice were anesthetized using isoflurane and injected intracranially in the right cerebral hemisphere by puncturing the cranium – 2 mm laterally of the sagittal suture using an intradural needle and a Hamilton syringe. MV (5 × 10^6 infectious units) suspended in 20 μL of Opti-MEM was inoculated. Each experimental group consisted of six mice.

**In vivo experiments for detection of tumor targeting and transgene expression.** Tumors were established by inoculating Raji cells (10^7/100 μL per site) into the right flanks of 6- to 8-week-old severe combined immunodeficient (SCID) mice (Harlan). When tumors measured 20 to 40 μL, mice received either five i.t. or five i.v. injections of MV-PNP HmutmutCD20 at 10^6 infectious units in 100 μL Opti-MEM (5 × 10^6 infectious units; virus titer determined on Vero-αHs cells), on days 6, 7, 8, 9, and 10 postimplantation. Six days after first injection, mice were sacrificed, and the tumors were harvested and halved. One half was dissolved in RNAlater buffer (Ambion). Levels of MV N mRNA in tumors harvested from each group (n = 4) were measured by quantitative reverse transcription-PCR (RT-PCR) as described previously (35). The other tumor half was used for PNP activity measurements.

**In vivo experiments for assessment of efficacy.** Raji cell and MCL xenografts were established and treated as described above. F-araAMP (250 mg/kg/dose) was injected i.p. on days 11, 12, and 13 after implantation. Control animals (mock therapy groups) were injected with equal volumes of Opti-MEM containing no virus. Each experimental group consisted of 10 mice. Tumor diameters were measured every 3rd day and the volume (product of 0.5 × length × width) was calculated as mean ± SE for each group. Animals were sacrificed at the end of the experiment, when tumor burden reached a volume of 1,500 μL (10% of body
weight). Animals that died without carrying large tumors (<1,500 μL) were excluded from the survival analysis; necropsy did not reveal drug-related symptoms.

**Statistical analyses.** Data were analyzed by using the ANOVA test to compare the treatment groups for tumor volume analysis. The two-sample t test was used to make pairwise comparisons between the treatment groups. Survival data were analyzed by the Kaplan-Meier method, and the log-rank test was used to test for significance between all the groups. Because only mice that had the event of interest were analyzed, the two-sample t test was also used to make pairwise comparisons between the groups. P values <0.05 were considered statistically significant and the JMP program version 6 was used for all analyses.

**Results**

**Generation and characterization of armed and targeted MV.** We generated a recombinant MV in which the attachment protein (H) was modified to ablate specific recognition of the natural

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**Figure 1.** Construction and characterization of armed and CD20-retargeted viruses. A, schematic drawing of a recombinant MV genome encoding a hybrid attachment protein (H) displaying an anti-CD20 single-chain antibody (scFv). The amino acid sequences of the linker flanking the scFv are indicated on the bottom line by one-letter code. HHHHHH, a six-histidine peptide displayed downstream of the scFv to allow infection of Vero-αHis cells via the αHis pseudoreceptor. The H protein indicated as “CD46/SLAM blind” has the following mutations: Y481A, R533A, S548L, and F549S for ablation of infectivity via the MV receptors CD46 and SLAM. An additional transcription unit coding for either the *E. coli* PNP gene or the EGFP gene, respectively, was inserted upstream of the N gene. B, expression of PNP and of the H and N viral proteins. For detection of PNP, Vero-αHis cells were infected at MOI 0.5 with MV-PNP (lane 2) or MV-PNP H blindantiCD20 (lane 3), respectively, and cells were lysed for immunoblotting 36 h postinfection. For detection of H and N, immunoblots of 5,000 infectious particles were done (lane 1, MVgreen; lane 4, MVgreenHblindantiCD20). C and D, infection of cells through the targeted receptors. C, cells were infected with MVgreenHblindantiCD20 (top row) or MV-PNP H blindantiCD20 (bottom row) at MOI 0.5 and photographed 36 h later. Scale bars, 100 μm. Bottom table, relevant receptors expressed by each cell line. D, primary mouse embryo fibroblast derived from transgenic CD46Ge mice were infected ex vivo with either MVgreenHblindantiCD20 or MVgreen, respectively, at MOI 0.5. Scale bars, 100 μm.
receptors CD46 (18) and SLAM (17), and fused to a single-chain antibody conferring selective entry through the B-lymphocyte antigen CD20 (ref. 15; Fig. 1A). To facilitate virus production, a COOH-terminal histidine hexapeptide was added to the MV HblindantiCD20 hybrid protein (21). To gain control over viral replication and to improve oncolytic properties, the E. coli PNP gene was inserted in the viral genome (Fig. 1A).

An armed virus (MV-PNP), an armed and targeted virus that does not recognize its natural receptors (MV-PNP HblindantiCD20), and a targeted virus expressing green fluorescent protein (MVgreenHblindantiCD20) were engineered. The characteristics of these viruses were confirmed by immunoblotting for PNP, or the viral proteins H and N. A band corresponding to PNP with an apparent molecular weight of 25 kDa was detected in cells infected with MV-PNP or MV-PNP HblindantiCD20, respectively (Fig. 1B, lanes 2 and 3). Reduced electrophoretic mobility of the hybrid H glycoprotein was confirmed for the MV expressing HblindantiCD20 (Fig. 1B, lanes 3 and 4). The N protein analysis confirmed equivalent incorporation of these proteins in particles of the standard and recombinant MV (Fig. 1B, lanes 1–4).

We next assessed the specificity of the retargeted viruses to enter human cell lines through different receptors. Cells expressing various combinations of natural MV receptors, or of targeted receptors, were infected at a MOI of 0.5 and monitored after 36 h for the appearance of syncytia and/or enhanced green fluorescent protein (EGFP) expression, reporting virus replication. As expected, retargeted MV entered cells exclusively through CD20 or the His-antibody pseudoreceptor (Fig. 1C, top row, second, fourth, and fifth panels).

To assess specificity in an ex vivo approach, and to confirm receptor expression, we used embryonic fibroblasts from transgenic CD46Ge mice that express CD46 with human-like tissue specificity (36). These CD46-expressing cells were susceptible to infection with MVgreen (Fig. 1D, right; syncytia formation and EGFP expression) but not with MVgreenHblindantiCD20 (Fig. 1D, left), as expected.

Dual prodrug function: early control of viral spread versus late enhanced cell killing. We then assessed whether produgs can be used to arrest viral spread in cells infected with MV-PNP HblindantiCD20. When MeP-dR was added to medium 12 h after infection at MOI 0.5, no syncytia were observed 36 h postinfection (Fig. 2A, bottom left). On the other hand, cells infected with MVgreen HblindantiCD20 showed characteristic cytopathic effects (CPE) with widespread syncytia formation, even in the presence of produg 36 h postinfection (Fig. 2A, right). Administration of MeP-dR alone up to a concentration of 100 µmol/L did not cause any CPE within 120 h (data not shown).

To document quantitatively the efficacy of produg in arresting spread of the armed MV, we measured the infectious particles produced by this virus and a control recombinant MV (MVgreen
enhanced the cytotoxic effect of MV-PNP without prodrug addition resulted in similar titers (Fig. 2B, third column), but when prodrug was added immediately after infection, no infectious particles were produced (Fig. 2B, fourth column; detection limit, 10 infectious units). Titers of this virus were reduced ~1,000 times when prodrug was added 12 or 24 h postinfection (Fig. 2B, fifth and sixth columns, respectively), suggesting that only secondary spread was completely inhibited. This is consistent with lack of syncytia formation and MeP-dR control of viral replication early during infection.

We also characterized the cytoreductive effects of different prodrugs. A model lymphoma cell line (Raji) expressing CD20 was infected with MV-PNP HblindantiCD20 at MOIs of 0.01, 0.1, and 1 (Fig. 2C). The prodrugs MeP-dR (left), or F-araAMP (center), or no drug (right) were added to the medium ab initio and cell viability was determined by MTT cell proliferation assay. Both prodrugs enhanced the cytotoxic effect of MV-PNP HblindantiCD20 compared with viral infection alone. Cytotoxicity enhancement was moderate at early infection times, but very strong after virus multiplication, accelerating complete cell lysis (Fig. 2C, red lines). As expected, F-araAMP without concomitant virus infection was toxic for Raji cells, but only after a long incubation time, whereas MeP-dR only initially slowed cell replication. Altogether, these data suggest that prodrug availability in the initial phases of infection results in selective elimination of the infected cells, whereas extensive prodrug toxification in later infection phases strongly accelerates lysis of all cells.

Early prodrug administration protects mice from lethal encephalitis. We then assessed in an animal system whether the MV-PNP/prodrug system can be used as a fail-safe feature. Toward this, we injected MV in the brain of IFN knockout mice expressing human CD46 with human-like tissue specificity. MV administration into the brain of these Ifnar<sup>−/−</sup>CD46<sup>Ge</sup> mice causes fatal meningoencephalitis (36).

Mice were infected intracranially either with $5 \times 10^5$ PFU MV-PNP, MV-PNP HblindantiCD20, or mock infected. One group received 250 mg/kg/dose F-araAMP i.p. for 3 consecutive days starting at 24 h after infection with MV-PNP. In a pilot experiment, mice were sacrificed 5 days postinfection and sagittal brain sections were characterized histologically (Fig. 3A). Section of MV-PNP–infected brain (without prodrug administration) disclosed meningitis with inflammatory infiltrates of leukocytes (Fig. 3A, top left, arrow). In contrast, infection with MV-PNP HblindantiCD20, or mock infection, or infection with MV-PNP followed by prodrug administration did not cause disease. The meninges were thin and the parenchyma was intact.

We then assessed whether prodrug administration protected mice from lethal encephalitis. Intracranial inoculation with MV-PNP caused lethal encephalitis in six of six infected mice: Clinical signs of neural diseases, including initial hyperactivity and awkward gait, were followed by lethargy, lack of mobility, and death 5 to 7 days after virus administration (Fig. 3B). In contrast, infection with MV-PNP was well tolerated when F-araAMP was administered i.p. for 3 consecutive days starting 24 h after viral infection. Thus, if administered early after infection, F-araAMP controls viral infection even in the brain. Inoculation with $5 \times 10^5$ PFU of a CD46-detargeted MV (MV-PNP HblindantiCD20) did not cause lethal infection, although certain animals infected with this virus showed mild clinical symptoms (data not shown). Thus, in this transgenic mouse model, MV replication was arrested by F-araAMP administered early after infection, and MV detargeting also eliminated viral spread and pathogenicity. Therefore, MV vectors armed with a prodrug convertase are fail-safe, at least in early infection phases, even in this very sensitive lethal encephalitis model. Moreover, detargeting/retargeting is an alternative effective way of enhancing vector safety.

Efficient oncolysis of Raji cell xenografts. Next, transgene expression and oncolytic efficacy of the armed and retargeted MV were characterized after s.c. implantation of Raji lymphoma xenografts in SCID mice. This established model for treatment of disseminated hematologic malignancies with MV (11) is not responsive to F-araAMP.

First, efficiency of tumor xenograft transduction was measured by quantitative RT-PCR after infection through different routes. On average, $7.6 \times 10^5$ MV N mRNA copies per $10^5$ cells (or 1 µg total

Figure 3. F-araAMP controls armed MV in vivo. Ifnar<sup>−/−</sup>CD46<sup>Ge</sup> mice (each group n = 6) received intracranial (i.c.) injection of MV-PNP (CD46-entry competent virus), MV-PNP with subsequent i.p. injection of 250 mg/kg/dose of F-araAMP for 3 consecutive days starting 24 h postinfection, MV-PNP HblindantiCD20 (CD46-entry incompetent virus), and mock, respectively. A, HE-stained sagittal brain sections obtained 5 d after viral inoculation. B, Kaplan-Meier survival curves of mice treated like described above.
RNA) were detected after i.t., and ~30 times lower levels after i.v. administration, of a total dose of 5 × 10⁶ PFU MV-PNP HblindantiCD20 (Fig. 4A). Second, transduction efficiency was estimated through PNP activity (activity was expressed as PNP units; 1 unit represents 1 nmol MeP-dR–converted/mg tumor cell extract/h): 118 ± 66 PNP units were measured after i.t. injection and ~15 to 20 times lower levels after systemic administration of MV-PNP HblindCD20 (Fig. 4B).

We then examined the effect on tumor growth of i.t. infection of MV-PNP HblindCD20 with or without subsequent i.p. administration of F-araAMP (Fig. 5A). Control groups were treated with mock or prodrug only, respectively, and tumor diameters were measured every 3 days. A strong oncolytic effect of the infection with MV-PNP HblindCD20 alone was documented, which could be minimally enhanced by prodrug administration. F-araAMP alone showed only a marginal effect.

Survival benefits are illustrated using Kaplan-Meier estimate with a 1,500 μL tumor volume defined end point (Fig. 5B). Administration of MV-PNP HblindCD20 in the presence of prodrug resulted in significant increase of survival compared with mock therapy or administration of F-araAMP alone (P < 0.001 for both by the two-sample t test). In this experimental approach, survival time was slightly extended by treatment with PNP vector plus F-araAMP compared with virus alone (two-sample t test P = 0.0343).
Because premature F-araAMP administration may interfere with viral replication and oncolytic efficacy, we tested two delayed application schedules. Four groups of 10 mice were treated with MV-PNP HblindantiCD20 as described above, and F-araAMP was administered either 1, or 8, or 15 days later. Thirty-five days after the last viral treatment, 8 of 10 animals in the group treated with prodrug at day 1 were alive, whereas only six and five mice survived when prodrug was administered at days 8 and 15, respectively (data not shown). Survival data were consistent with prodrug administration directly following the 5 days viral treatment being most effective in this Raji lymphoma xenograft model.

Systemic administration of the armed and targeted MV extends mouse survival. Because i.t. therapy is of limited value for the treatment of disseminated malignancy, we tested the armed and CD20-targeted MV in Raji cells xenografts after systemic administration via tail vein (Fig. 5C). Here, treatment with solely F-araAMP or virus alone did not have a statistically significant effect compared with mock treatment (P > 0.05 from two-sample t test), but the combination of MV-PNP HblindCD20 plus F-araAMP had a therapeutic effect compared with mock treatment, F-araAMP, and virus alone (P < 0.001 from two-sample t test), and was highly synergistic (P = 0.0038; interaction test from two-factor ANOVA model). The mean tumor volume at day 26 after implantation was about half the mock-treated tumor volume (P < 0.001). This was reflected in extended survival (Fig. 5D). Prolongation of survival was significant after combination of both prodruig and virus (log-rank test P = 0.001) and showed synergistic effects (P = 0.0196; interaction test from two-factor ANOVA model). Thus, systemic administration was less efficient than i.t. treatment, allowing documentation of synergistic effects of virus and prodrug.

Oncolytic efficacy was maintained in part after systemic inoculation, even if PNP expression levels in tumors were 15 to 20 times lower than after i.t. inoculation (Fig. 4B). A more homogenous virus distribution in the tumor after systemic inoculation may account for this enhanced effect: A homogenous mixture of 0.1% PNP-expressing cells and 99.9% cells not expressing PNP in a tumor elicited substantial antitumor effects (37).

Complete but temporary regression in a MCL tumor model. We assessed oncolytic efficacy in a second model. We selected an aggressive lymphoma model, Granta 519 cells (30, 38) implanted these MCL xenografts, and administered virus and prodrug as in the Raji cell xenograft model.

Twenty-five days after i.t. treatment with MV-PNP HblindantiCD20 alone, or combination therapy, all tumors of all the mice in both groups (in total 20 tumors) became undetectable (Fig. 6A). However, in a majority of the mice, tumors reappeared after a few weeks. Survival after 40 days was 100%, and after 60 days was 60% (Fig. 6B). In average, treated mice survived two to four times longer than untreated mice. F-araAMP treatment alone led to marginal tumor growth retardation and extended survival slightly. Administration of F-araAMP directly after i.t. viral treatment did not enhance the oncolytic efficacy of the virus. Thus, in this aggressive lymphoma model, virotherapy was highly effective.

Cells of MCL patients are permissive for infection. To assess infection efficiency and to test the feasibility of viral transduction in an ex vivo approach, peripheral blood cells from one MCL patient were collected and infected with MVgreen HblindantiCD20. Figure 6C shows a microscopic analysis of these cells: EGFP expression, reporting virus transduction, was observed in large groups of cells visualized within the even larger clumps forming in this culture.

Surgical samples from the spleens of two other patients were also inoculated with MVgreen HblindantiCD20. In these samples, 6% and 1% infected cells were detected, respectively (data not shown;
because enhanced clumping resulting in exclusions from the FACS analysis is a major issue with infected cell cultures, the percentiles of infected cells may have been underestimated). As control, another vector targeted to enter cells through the carcinoembryonic antigen was used. This virus did not infect these cells (data not shown). Thus, primary cells of MCL lymphoma patients are permissive for MV infection.

Discussion

We armed MV with a prodrug convertase activating the approved lymphoma chemotherapeutic fludarabine, and retargeted it to the B-cell specific antigen CD20. Therapeutic efficacy of this vector, and synergy with F-araAMP after systemic administration, were shown in a Burkitt’s lymphoma xenograft model. In a model of MCL, i.t. virus treatment alone consistently caused tumor regression, and supported 2- to 4-fold longer animal survival. Adaptation of this prodrug/convertase system to MV also provides a safety feature.

Combination treatments are well established in cancer therapy, with the FCR regimen for selected low-grade NHL lymphoma being a relevant example. The first approved oncolytic therapy regimen combines an oncolytic adenovirus with chemotherapy (7). In preclinical trials, oncolytic viruses have been combined with established chemotherapeutic drugs like cisplatin, vincristine, or doxorubicin (39). These dual-mode therapies resulted in additive or synergistic effects compared with either therapy alone.

The rationale for these combination therapies has been the lack of expected cross-resistance between the oncolytic virus and the chemotherapeutic, rather than functional, reinforcement achieved in our protocol when fludarabine is modified locally through the production of highly diffusible 2-fluoroadenine (29). Our study redefines the chemovirotherapy concept by introducing synergy in the form of a targeted virus functioning locally as an amplifying agent.

Preclinical data have already shown the efficacy of other virotherapy approaches based on viral expression of prodrug convertases. For example, a herpes simplex virus (HSV) expressing cytochrome P450 oxidase augmented the effect of cyclophosphamide (40). An adenovirus expressing uracil phosphoribosyltransferase enhanced the effect of 5-fluorouracil locally, which was necessary to overcome chemoresistance to this drug in the treatment of biliary tract cancer (41).

Prodrugs have also been used to control the replication of another set of oncolytic viruses expressing convertases: For example, GCV arrests replication of HSV or of adenoviruses coding for HSV-tk (42, 43). Timing of prodrug application is critical when the aim is termination of virus infection: In our study, F-araAMP arrested spread of an MV-expressing PNP when administered 24 h after a potentially lethal challenge, and completely suppressed disease symptoms. These experiments suggest that F-araAMP can be used in the clinic as a fail-safe system to control the spread of MV-based oncolytic vectors, but only in early infection phases.

Timing of prodrug application is also important when the aim is therapeutic efficacy. Increasing the interval between infection and prodrug administration in a chemovirotherapy approach improved therapeutic efficacy of an armed adenovirus (41) because the virus can spread further in the tumor before the prodrug is added to enhance local cell killing. In our study, the most effective time point for prodrug administration after MV-PNP i\textsuperscript{t} antiCD20 i.t. treatment of Raji xenografts was immediately after the last virus injection. Other tumor types and routes of virus administration might require different schedules.

Ideally, the prodrug should be administered when replication is at its peak; however, it has not been possible to monitor viral replication in patients. The development of recombinant MV that express plasma proteins reporting replication (44) allows noninvasive monitoring of MV infection in clinical trials. Thus, planning of accurate prodrug administration schedules is now possible based on these tracking tools.

In summary, our experiments indicate that a PNP-armed and CD20-targeted MV can synergize with fludarabine in lymphoma therapy. This vector has the potential to substitute Rituximab in an improved FCR therapeutic regimen, or to be combined with fludarabine or cyclophosphamide, the other two components of the regimen. Cyclophosphamide immunosuppression enhances MV oncolytic efficacy in an immunocompetent host (45), as it does with oncolytic reoviruses, HSVs, and adenoviruses (46–48). Toward clinical trials, more data on timing of vector application in combination with these two drugs are currently sought.

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Chemovirotherapy for Lymphoma

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Lymphoma Chemovirotherapy: CD20-Targeted and Convertase-Armed Measles Virus Can Synergize with Fludarabine

Guy Ungerechts, Christoph Springfeld, Marie E. Frenzke, et al.


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