Current problems and possible solutions in the treatment of nasopharyngeal carcinoma in Indonesia
Wildeman, M.A.M.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 8

Cytolytic virus activation therapy for Epstein-Barr virus driven tumors

Maarten A. Wildeman,
Zlata Novalic,
Sandra A.W.M. Verkuijlen,
Hedy Juwana,
Alwin D.R. Huitema,
I. Bing Tan,
Jaap M. Middeldorp,
Jan Paul de Boer,
Astrid E. Greijer


http://clincancerres.aacrjournals.org/content/18/18/5061
Abstract

Purpose

Nasopharyngeal carcinoma (NPC) is causally linked to Epstein–Barr virus (EBV) infection. Because all tumor cells carry EBV, the virus itself is a potential target for therapy. In these tumor cells, EBV hides in a latent state and expresses only a few non-immunogenic proteins for EBV maintenance and contributes to tumor growth. We developed a cytolytic virus activation (CLVA) therapy for NPC treatment, reactivating latent EBV, triggering immune recognition, and inducing susceptibility to antiviral therapy.

Experimental Design

CLVA therapy combines gemcitabine (GCb) and valproic acid (VPA) for virus activation and tumor clearance with (val)ganciclovir (GCV) as the antiviral drug to block virus replication and kill proliferating virus-infected cells. CLVA treatment was optimized and validated in NPC cell lines and subsequently tested in 3 Dutch patients with NPC that was refractory to conventional treatment.

Results

In NPC cell lines, both GCb and VPA can induce the lytic cycle of EBV. Their combination resulted in a strong synergistic effect. The addition of GCV resulted in higher cytotoxicity compared with chemotherapy alone, which was not observed in EBV-negative cells. CLVA therapy was analyzed in 3 patients with end-stage NPC. Patients developed increased levels of viral DNA in the circulation originating from apoptotic tumor cells, had disease stabilization, and experienced improved quality of life.

Conclusions

Our results in the initial CLVA-treated patients indicate that the therapy had a biological effect and was well tolerated with only moderate transient toxicity. This new virus-specific therapy could open a generic approach for treatment of multiple EBV-associated malignancies. Clin Cancer Res; 18(18); 5061–70. ©2012 AACR.
Introduction

Epstein–Barr virus (EBV) is causally linked to multiple cancers, including several lymphomas, undifferentiated nasopharyngeal carcinoma (NPC), and approximately 10% of gastric cancers worldwide. Although NPC is an uncommon disease in most countries, NPC is the 4th most common tumor among males in Indonesia and has a high incidence in southern China, northern Africa, and Alaska (1). Early diagnosis is essential because disease-free survival declines for patients with late-stage NPC (2). Unfortunately, early symptoms for NPC, such as epistaxis and tinnitus, are nonspecific and the majority of patients come to the hospital with advanced stage disease. In Indonesia, 87% of patients have NPC stage III or higher and 17% have distant metastasis at first presentation (3).

Because all NPC tumor cells harbor EBV, this virus itself is a possible target for therapy. In these tumor cells, EBV “hides” in a latent state due to methylation of the viral promoters, expressing only a few non-immunogenic viral proteins that are essential for EBV maintenance and contribute to tumor growth (4). This state of latency enables NPC tumors cells to evade the immune system, even in the presence of an existing strong immune response to multiple viral antigens (5, 6). Recent studies have shown that the lytic cycle of EBV can be efficiently induced by using chemotherapeutic agents effecting DNA synthesis and drugs affecting host DNA methylation and histone deacetylation (HDAC; refs. 7–11). The reactivation of EBV leads to the expression of proteins involved in viral genome replication and formation of new virions. These newly expressed proteins are highly immunogenic and could induce a powerful immune response toward the tumor cells containing the reactivated virus (5). In addition, lytic induction enables expression of viral kinases sensitizing the cells to antiviral treatment (9).

Inducing the lytic phase with chemotherapy in combination with a HDAC inhibitor caused reduced tumor volume in EBV-positive tumor cells in a mouse lymphoma model (8). The addition of an antiviral component to this combination therapy did further decrease tumor development. This concept was first used in 1998 for the treatment of a patient with EBV-positive lymphoma in which a combination of an HDAC inhibitor with antiviral therapy was administered (12). This was followed by a study using valproic acid (VPA) instead of arginine butyrate (13). The combination of chemotherapy (5-FU) with an HDAC inhibitor (HDACi) for a stronger induction of the lytic cycle was evaluated in a Dutch patient with end-stage NPC. After 5 days, the patient received antiviral treatment with valganciclovir (GCV). This treatment resulted in an increase of viral DNA in the circulation, reflecting shedding of apoptotic fragments from the tumor, which was not observed before treatment (14).

In this study, a novel combination therapy was developed targeting EBV within the tumor cell by combining EBV reactivation with subsequent antiviral therapy. After validation in a single naturally EBV-infected NPC cell line and EBV-positive gastric cancer cell lines, this cytolytic virus activation (CLVA) therapy, comprising gemcitabine (GCb), VPA, and GCV, was applied as a new treatment modality in patients with EBV-positive NPC for which no curable treatment options were available. In addition to clinical parameters, biological response was analyzed by EBV viral load and serology.

Materials and Methods

Cell lines

The EBV-positive NPC cell line C666.1 was cultured in Dulbecco's Modified Eagle's Medium (DMEM; BioWitthaker) containing 10% fetal calf serum (FCS) and 1% penicillin/ streptomycin/glutamine (P/S/G). Cell culture plates were first coated with fibronectin (Calbiochem) in PBS (100µg/mL) for 1 hour at roomtemperature (rT). Gastric
carcinoma cell lines of AGS and the EBV-positive cell line AGS-BX1 were cultured in F12HAMEBioWitthaker) containing 10% FCS and 1% P/S/G. AGS-BX1 cells were cultured under G418 (geneticin; Life Science Technologies) selection. The AGS-BX1 harbors the EBV genome with an insertion of the neomycin-resistance gene and the GFP gene that disrupts the TK gene (kindly provided by L. Hutt-Fletcher).

**Western blot analysis**

Protein samples were obtained by lysing cells in a RIPA buffer in the presence of a protease inhibitor cocktail (Roche) for 30 minutes at 4°C. Total protein concentration was determined with the BCA protein assay kit (Thermo Scientific). Proteins were mixed with 4× sample buffer and boiled for 5 minutes at 95°C. Protein samples were run on SDS-PAGE and transferred to nitrocellulose sheets by blotting. Immunoblots were blocked with 5% dried milk powder in PBST (0.05% Tween20 in PBS) and incubated with the primary antibodies BZ-1 for detecting ZEBRA (a gift from P. Farrell) and actin-HRP (C4; Santa Cruz). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (DAKO) the proteins were visualized by the ECL detection kit (GE Healthcare) according to instructions provided in the manual.

**Immunofluorescence**

C666.1 cells were cultured on 10-mm coverslips in 24- well plates. Cells were induced with 3 μmol/L GCb and 0.3 mmol/L VPA for 2 days. Cells were fixed by incubation with methanol-acetone for 10 minutes. Subsequently, nonspecific binding was blocked by incubation in PBS containing 10% FCS. Glass slides were incubated with BZ1 antibody and, after washing with PBS containing 0.05% Tween-20, incubated with the secondary antibody conjugated with fluorescein isothicyanate (FITC). Slides were mounted in Vectashield (Vector Laboratories Inc.) containing 0.3 μmol/ L 4',6-diamidino-2-phenylindole (DAPI). Immunofluorescence was analyzed by using a Leica microscope.

**Cytotoxicity analysis**

For CLVA treatment cytotoxicity screens, 25,000 C666-1 cells were seeded in a 96-well plate in 100 μL medium and were allowed to adhere for 24 hours at 37°C in5% CO₂. The next day, serial dilutions of GCb in the presence of 0.3 mmol/L VPA were added to cells in triplicate for 6 days. After drug incubation, 5 μL of MTT (5 mg/mL; Roche) was added to each well and the mixture was incubated for 3 hours. Thereafter, 100 μL of isopropanol–HCl solubilization buffer was added and the plates were incubated at 37°C overnight. The OD₅₉₅ was determined using a microplate reader (Molecular Devices).

**Patients**

Three patients with histologically confirmed residual, recurrent, and metastatic EBV-positive NPC were included in this study after they had failed conventional curative treatment options and were deemed incurable. All patients signed an informed consent for this experimental pilot study. Before treatment, patients had a strong and broadly reactive humoral immune response against EBV antigens, measured by ELISA and immunoblot as described below.

**CLVA therapy**

One treatment cycle extends over 42 days. Patients received GCb (1250 mg/m²; Fresenius Kabi Oncology Plc) on days 1 and 8 intravenously. Valproic acid (generic medicine) was administered orally (12.5 mg/kg per day) during the first 14 days of the treatment cycle. From days 9 to 22, patients were treated with GCV (Roche) daily (3 × 450 mg/day orally). This treatment cycle of 42 days was repeated 6 times.
Clinical monitoring of patients

All patients had a baseline magnetic resonance imaging (MRI) scan of the head-and-neck region, an ultrasound of the neck region, and complete body PET-CT scan. After every 2 treatment cycles, the tumor response was measured by imaging. In case of response or stable disease, patients received a total of 6 cycles. For the nasopharyngeal and neck region, MRI was carried out. Distant metastases in the thoracic region were monitored with CT-imaging. After finishing the treatment, a PET-CT scan was conducted. Adverse events were graded on the basis of the National Cancer Institute Common Toxicity Criteria version 3.0. Blood samples for measuring EBV viral load and humoral antibody response were obtained every week. Brush sampling for measuring EBV DNA load was carried out every 3 weeks.

Nucleic acid isolation

DNA was isolated from nasopharyngeal brushing samples by silica-based nucleic acid extraction as described previously (15). One milliliter of lysate was used as input for the isolation procedure, and the nucleic acids were eluted in 100 µL water. Reagents for the isolation procedure were obtained from BioMerieux.

RNA was isolated from cell lines by addition of 250 µL TRIzol to 300,000 cells according to the manufacturer’s instructions (Invitrogen).

EBV viral load by real-time quantitative PCR

The EBV DNA load in whole blood was determined by a quantitative LightCycler480 amplifying a 99-bp part of EBNA1. The probe master mix used was supplemented with primer QP3 and 4, and hybridization probes were used for quantification as described previously (16). Real-time PCR (RT-PCR) reagents were obtained from Roche Diagnostics. Then, 10-fold serial dilutions of spectrophotometrically quantified plasmid DNA containing the EBNA1 target sequence were used to create a standard curve. To check for putative inhibition of PCR, EBV DNA-negative samples were spiked with 1,000 copies of EBV plasmid DNA. β-Globin PCR was carried out with the primers PCO3 (5'-ACACAACGTGTTCACTAGC-3') and PCO5 (5'-GAAACCCAAGAGTCTTCTCT-3'), which generate a 209-bp PCR product. Reaction conditions were as described previously (17). Quantification of the amplification products was carried out with the second derivative software of the LC480 (Roche).

cDNA synthesis and quantitative RT-PCR

RNA was treated with 1 µL RQ RNase-free DNase (Qiagen), 1 µL RQ1 RNase-free DNase 10× Reaction Buffer, and precipitated with 1 µL 3 mol/L sodium acetate, 0.5 µL linear acrylamide (Ambion; 5 mg/mL), and 25 µL of 100% ethanol at –80°C. Precipitated RNA was reverse transcribed by gene-specific cDNA synthesis using a multiprimered approach for 10 minutes at 65°C. Subsequently, 2 µL RT buffer, 10 µL dNTPs (2 mMol/L), 2 µL DTT (100 mmol/L), 0.5 µL H₂O, 0.25 µL RNasin, and 0.25 µL AMV reverse transcriptase was added to each sample and incubated for 1 hour at 42°C. EBV gene expression was quantified by quantitative LightCycler PCR using an LC480 system (Roche). Quantitative PCR was carried out using a LightCycler 480 SYBR Green I Master kit (Roche) in a total reaction mixture of 10 µL containing 2.5 µL of 10-times-diluted cDNA, 5 µL 2× LightCycler 480 SYBR Green Master Mix, 0.5 µL (10 pmol/µL) of the gene specific primers, and 1.5 µL H₂O. Primers used in the quantitative LightCycler PCR are listed in supplementary Table S1. Absolute quantification was determined using a standard curve of the plasmid pool containing all targets for quantifying the exact amount of RNA molecules for each target.
Serology

Humoral antibody responses were measured in the serum of the patients with NPC. Immunoglobulin A (IgA) reactivity was assessed by a synthetic peptide-based ELISA using immunodominant epitopes derived from EBNA1 and VCA-p18 as described previously (18). Immunoblot analysis was carried out on blot strips containing HH514.c16 nuclear antigen induced by TPA and sodium butyrate to produce the late lytic phase of EBV. Strips were prepared and analyzed as described previously (19, 20).

Results

Lytic induction by GCb in combination with VPA in vitro

To validate the concept of CLVA therapy, the combination of a chemotherapeutic agent and a histone deactylation inhibitor was tested on human EBV-positive NPC (C666.1) and gastric carcinoma (AGS-BX1) cell lines. C666.1 cells carry a tightly latent EBV, whereas AGS-BX1 cells are less latent, showing spontaneous lytic EBV reactivation in approximately 5% of cultured cells. For inducing the EBV, replicative phase cells were treated with 3 µmol/L GCb and 0.3 mmol/L VPA, either separately or simultaneously. Expression of lytic RNA and proteins was analyzed by quantitative RT-PCR and Western blot (Fig. 1). The ZEBRA protein, acting as the EBV lytic switch, is upregulated marginally by the HDAC inhibitor VPA alone in C666.1 cells. The addition of GCb to VPA resulted in a stronger lytic induction, as indicated by the increased level of ZEBRA protein (30-fold) in C666.1 cells and a 3-fold increase in the AGS-BX1 lines, which already showed detectable ZEBRA expression at baseline.

The combination GCb and VPA resulted in a steep increase in ZEBRA RNA levels in both the C666.1 (150-fold) and AGS-BX1 cell line (10-fold; Fig. 1C) and was dosedependent for each drug (Fig. 2). Other EBV lytic cycle genes besides the ZEBRA switch protein, such as protein kinase (PK), thymidine kinase (TK), and the structurally small capsid protein VCA-p18, were also induced by GCb and VPA in a dose-dependent manner in C666.1 cells (Fig. 2). To analyze the percentage of cells entering the lytic phase, the ZEBRA protein was stained in C666.1 cells induced for 2 days by GCb and VPA. More than 80% of the cells showed ZEBRA expression in a typical staining of nuclear dots (Fig. 3). The intensity of the staining varied among the cells; however, in more than 10% of cells, a strong immunofluorescence was observed.

Cytotoxicity of lytic induction increased by antiviral treatment

Induction of the EBV lytic cycle can create susceptibility to antiviral treatment and consequently increases antiviral drug-induced cytotoxicity. In the early phase of EBV reactivation, the enzymes TK and PK are expressed, which convert the antiviral drug, ganciclovir, into its cytotoxic form (9, 21). The C666.1 cell line was used to induce the lytic phase of EBV by adding 0.3 mmol/L VPA and a serial dilution of GCb (Fig. 4A). After 3 days, the cells were reseeded and analyzed for cytotoxicity in the absence and presence of 20 µmol/L GCV. Cytotoxicity (MTT) assays were conducted 6 days after initial culture and the increase in cytotoxicity caused by the addition of GCV to GCb was determined. The IC_{50} values for GCb shifted under the addition of GCV from 6.1 to 3.1 mmol/L. In AGS-BX1, the addition of GCV lowered the IC_{50} values of GCb from 4.3 to 2.0 µmol/L (data not shown). The increased cytotoxicity provided by GCV was not very high, but GCV showed a clear effect on inhibition of EBV replication. We observed a total block in production of RNA encoding the viral capsid protein VCA-p18 above the 7.5 mmol/L GCV level (Fig. 4B). The absence of VCA-p18 reflects the inability of induced EBV to generate new virions, thus blocking virus spread.
Figure 1 | Lytic induction in cell lines. EBV-carrying NPC (C666.1) and gastric carcinoma (AGS-BX1) cell lines were cultured in the presence of gemcitabine (GCb), valproic acid (VPA), and a combination of the 2 drugs. Lytic induction was indicated by: A, increase of ZEBRA protein on Western blot; B, quantification ZEBRA protein; and C, quantification of ZEBRA mRNA levels by RT-PCR.
Figure 2 | Dose-dependent induction of EBV lytic cycle by GCb and VPA in C666.1 cells. Dose escalation of GCb was analyzed for lytic induction. A, RNA levels of lytic genes (ZEBRA, early genes TK, PK, and late gene VCA-p18 gene) were determined and normalized to a cellular housekeeping gene. B, Western blot analysis of the level of the proteins expressed from the immediate early gene ZEBRA was assessed with the loading control ß-actin. Dose escalation of GCb was analyzed for lytic induction after addition of 0.3 mmol/L VPA. C, RNA levels of lytic genes were determined and normalized to a cellular housekeeping gene. D, analysis and quantification of the ZEBRA protein levels was measured on Western blot assay. Dose escalation of VPA in presence of 3 µmol/L GCb was studied. E, RNA levels of lytic genes were determined and normalized to a cellular housekeeping gene. F, analysis and quantification of the ZEBRA protein levels was measured on Western blot assay.
Cytolytic virus activation therapy for Epstein-Barr virus driven tumors

Figure 3 | Percentage of lytic-induced C666.1 cells by GCb VPA was visualized by ZEBRA protein staining. A, the percentage of ZEBRA-positive cells in untreated condition and after incubation for 48 hours with 3 µmol/L GCb and 0.3 mmol/L VPA. B, typical example of the punctuated ZEBRA staining in the lytically induced C666.1 cells (left), with the DAPI nuclear control (right). Although ZEBRA expression is (weakly) induced in most cells, brighter nuclear ZEBRA staining is observed in approximately 11% of the cells in a typical experiment.

**CLVA therapy in patients with end-stage NPC**

**Patient 1**

The first patient treated with CLVA therapy was a 48-year-old female (WHO performance status 1). She had a local recurrence with extension into the retropharyngeal recess surrounding the internal carotid artery. Twenty-two months before commencement of the CLVA treatment, she received chemoradiation for T4N1M0 NPC. Fifteen months after initial treatment, she received photodynamic therapy for local recurrence in the nasopharynx, but only had a partial response. This patient received a total of 6 cycles of CLVA therapy. During the first 2 cycles patient received 1.25 mg/kg VPA instead of 12.5 mg/kg due to logistic failure.

**Tumor response**

The patient showed a significant decrease of tumor in the nasopharynx after 2 courses (Fig. 5A and 5B). During the remaining treatment and for up to 11 months of the follow-up period, the patient had stable disease. An MRI scan after treatment showed disease progression of the tumor in the nasopharynx (Fig 5B).
Adverse events

This patient developed a grade 4 toxicity with platelet count decrease after course 3, but recovered completely before the start of course 4. After the first GCb course, the patient again developed a grade 3 toxicity platelet count; therefore, she received 50% of the GCb dose for the remaining courses. Due to grade 3 toxicity of hemoglobin levels, the patient received blood transfusions.

Patient 2

A 52-year-old male (WHO performance status 0) patient was referred to our hospital with disease progression after palliative radiotherapy for a T2aN3M1 NPC diagnosed 10 months previously. The disease progression consisted of cytologically proven positive lymph nodes high in the mediastinal region and a costal metastasis. In addition, 2 regional lymph nodes in the left neck were assessed and found to have persistent tumor after radiotherapy. This patient received a total of 6 cycles, with no dose reductions. Blood sampling for EBV viral load and antibody response were collected every 2 weeks.

Tumor response

After 2 courses of CLVA therapy, patient 2 was observed to have responded with a reduction of one of the tumor positive lymph nodes in the neck to normal size on CT-imaging. After 6 cycles, imaging showed the absence of PET activity. The remaining neck-node tumor remained stable after 6 courses of treatment. As there was an increase in quality of life and improvement of well-being, patient 2 received another course of
adjuvant radiotherapy on the residual positive lymph node (36 Gray) in the left neck region and has been observed to have a persistent stable disease for 6 months in the follow-up period.

**Adverse events**

Patient 2 did not encounter any grade 3 or 4 adverse events.

**Patient 3**

The 3rd patient was a 48-year-old female (WHO performance status 1) with progressive disease in the nasopharynx, which was histologically proven as recurrent disease, and a cytologically proven lymph node in level V of the neck region. In addition, PET-CT showed supraclavicular, intrathoracic, and left thyroid lobe metastases. Nine years previously, she underwent a modified radical neck dissection for a tumor in the neck with an unknown primary site. This patient had already received palliative treatment with capecitabine and radiotherapy before initiation of the present treatment with CLVA. Patient received a total of 5 treatment cycles, with 25% dose reductions during course 5. The patient refused to undergo cycle 6.

**Tumor response**

This patient had a significant decrease of tumor mass in the nasopharyngeal region (Fig. 5C and 5D). The lymph nodes and distant metastasis remained stable for 6 months during the follow-up.

**Adverse events**

At the start of therapy, patient 3 had a grade 3 anorexia for which she received tube feeding during all courses of therapy and during follow-up. Patient 3 developed aspiration pneumonia grade 3 after cycle 4. During the 5th cycle, this patient was admitted to the hospital twice with two grade 3 infections. During the 5th course, she also suffered a grade 3 hepatotoxicity (increased SGPT).

**EBV DNA load in the tumor cells and whole blood of CLVA-treated patients with NPC**

The presence of high levels of EBV DNA in minimally invasive brushings, taken from the nasopharyngeal mucosa has been described to correlate with the presence of viable NPC tumor cells (22). In contrast, EBV viral DNA detected in the circulation of NPC patients may originate from apoptotic tumor cells rather than viable circulating tumor cells (29). The 3 patients treated with CLVA therapy were monitored for EBV viral load in nasopharyngeal brushing samples and in whole blood, drawn before, during, and after CLVA treatment (Fig. 6). Patients 1 and 2 did not have a visible tumor in the nasopharyngeal mucosa and, therefore, brushing samples were taken at longer intervals. The 3rd patient did have a locally visible tumor in the nasopharyngeal region and brushing samples were obtained every 2 weeks. The viral load measured in patient 3 before treatment was $4.3 \times 10^6$ EBV copies/brushing sample. After an initial 200-fold decrease in viral load during the second cycle of treatment down to $2.4 \times 10^4$ EBV copies/brushing sample, the viral load showed a rise again mainly during a prolonged interval in the treatment schedule. Brushing samples of the other CLVA-treated patients showed an occasionally positive result up to $10^4$ copies/brushing sample.
The viral DNA levels in blood were monitored to analyze the biological response to therapy. Before treatment, all patients had EBV copies below the clinical cutoff level, that is, $1.6 \times 10^3$ copies/mL of blood. At the start of therapy, patient 3 had slightly higher levels ($1.1 \times 10^4$ copies/mL of blood). Viral load in blood during CLVA treatment showed a highly dynamic response, probably reflecting tumor apoptosis. The highest fluctuations were observed in patient 1, in whom it varied from below detection level up to $1.4 \times 10^6$ EBV copies/mL of blood. In patients 2 and 3, lower EBV DNA levels were measured in the blood. A common pattern in the blood that was observed for all 3 patients was the appearance of viral DNA in the periods following treatment (Fig. 6).

**Humoral immune responses of CLVA-treated patients with NPC**

The IgG response against immunodominant EBV epitopes was analyzed. The data confirmed that the patients had a molecularly diverse anti-EBV immune response before treatment, including reactivations against lytic viral antigens. A potent immune response is needed to allow identification of therapy-induced new immunogenic viral proteins. Two patients showed a strong immune response against a wide diversity of viral proteins (Fig. 5E). The first patient had a weaker response, mainly directed against ZEBRA and VCA-p18. The
level of the immune response remained the same after treatment and the pattern of proteins recognized did not change.

Further, the level of the humoral immune response was determined by standardized peptide-based ELISA before and during therapy. High constant levels of VCA-p18 reactivity were observed, whereas the EBNA1 responses remained at a constant low level (data not shown).

Figure 6 | Effect of CLVA treatment on the viral load in whole blood (black line) and nasopharyngeal brushings (gray line) of the CLVA NPC-treated patients. In the boxes above the individual graphs, the therapy schedule is depicted. The arrows indicate the GCb infusions, 1250 mg/m². However the last 3 GCb infusions in patient 1 comprised only half of the dose. The VPA was given at a concentration of 12.5 mg/kg per day, except for the first 4 cycles for patient 1, wherein 1.25 mg/kg per day was administered. The GCV concentration was 3 × 450 mg/day and administered at the indicated times. DNA load was determined in blood samples obtained weekly and in the tumor cells obtained by NP brushing at the indicated time points.
Discussion

A new therapeutic approach was explored on the basis of reactivation of the latent viral genome in combination with antiviral therapy to target EBV within NPC tumor cells. This CLVA therapy proved to be effective \textit{in vitro} and, subsequently, CLVA therapy was administered to 3 patients with end-stage NPC disease. CLVA treatment in patients with end-stage NPC was well tolerated and showed a clinical response and improvement in quality of life during and after therapy in all patients. Side effects of CLVA therapy were transient and only moderate. These first \textit{in vivo} results indicate that the concept of epigenetic induction of viral antigens with subsequent administration of antiviral therapy may be a potent approach in patients with EBV-positive NPC, which can possibly be extended to other EBV-driven malignancies.

Gemcitabine is one of the most effective single-agent therapies in the treatment of NPC (23), and was recently studied in 31 patients where initial platinum-based chemotherapy failed and subsequent GCb therapy resulted in partial response in 43.8\% of the patients and stable disease with minimal side effects in 28.1\% of patients (24). Besides tumor cell apoptosis induced by GCb, it could reactivate EBV within the tumor cells. The potential for EBV lytic induction by GCbin EBV-positive NPC cell lines was shown to be higher than by 5-FU, which was used in a previous juvenile NPC case study (14, 25, 26). Activation of highly methylated DNA of latent EBV can be enhanced by epigenetic chromatin remodeling of the EBV genome induced by HDAC inhibitors and DNA-demethylating agents (7–11). In the naturally EBV-carrying infected NPC cell C666.1, the combination of histone deactylase inhibitor VPA with GCb showed a clear synergistic action on the expression of EBV lytic genes, ZEBRA, TK, PK, and VCA-p18, which was confirmed in the gastric carcinoma cell AGS-BX1 harboring a recombinant EBV (Figs. 1–3). Lytic induction sensitized the EBV-positive cell lines to the antiviral drug ganciclovir (GCV), which resulted in an increase of cytotoxicity compared to the toxicity induced by GCb and VPA alone (Fig 4A). Further, EBV lytic gene expression is known to result in an increase of apoptosis in combination with GCV (27). This additional toxic effect was observed in an EBV-positive lymphoma animal model (25). In addition, enhanced toxicity by GCV was observed in a recent study by Sides and colleagues in a study wherein they provoked lytic induction with low-dose arsenic (28). Importantly, GCV resulted in reduced production of RNA encoding the late viral capsid antigen p18 (VCA -p18) in lytically induced C666.1 cells (Fig. 2). This clearly demonstrates the effect of GCV treatment on inhibiting new viral particle formation after lytic induction, which is necessary for the safety of CLVA treatment in patients with NPC.

The choice of drugs already approved for human use resulted in the rapid translation of CLVA-based treatment into the clinic. Therapy was administered to 3 patients with end-stage NPC and was well tolerated. We observed some toxicity, mainly consisting of neutropenia, in only one case. However, previous chemotherapy administered to this patient may be more causal to the reduced bone marrow capability to recover from GCb. The other 2 patients did not encounter any detectable side effects of CLVA therapy.

All 3 patients showed a biological response to CLVA therapy indicated by an increase in viral DNA load in the blood, as observed in a previous case study (14). This increase reflects fragmented EBV DNA derived from apoptotic tumor cells (29). The dynamics of theDNAload in all patients showed a similar trend, that is, an immediate decline of EBV DNA loads during therapy cycles and an increase in EBV DNA load in the recovering periods. A decline of EBV load in blood has been described to predict a good clinical response in patients with NPC (30). The unexpected increase of viral load in the period without treatment could reflect the functional recovery of the immune system and its effect in eliminating lytic induced cells. We failed to detect an increase in anti-EBV immune reactivity as result of the CLVA therapy, although more detailed analysis may be needed for this, including analysis of T-cell responses.
A residual (recurrent) tumor in the nasopharynx was only present in the 3rd patient. Viral load in noninvasive NP brushings taken regularly during and after therapy decreased, but did not disappear completely. Repeated biopsies in the nasopharynx during and after treatment were considered too invasive; therefore, it is unclear whether these viral loads represent shed tumor cells or viral reactivation in the nasopharynx.

Despite the small number of patients in this pilot study, which may prevent strong conclusions, we followed the clinical outcomes of these patients. All patients initially having progressive disease developed stable disease during and after treatment, and, in addition, a clear improvement of their clinical condition was observed.

In conclusion, virus-specific CLVA tumor therapy may provide a new and generic approach for treatment of multiple EBV-associated malignancies in both developed and developing countries worldwide. The 3 patients with endstage NPC all had a clinical response to CLVA therapy and an improved quality of life. A phase I/II trial was started recently (Eudract nr: 2010-022444-20). In this trial, additional PBMCs will be collected to obtain more insight in the immunological aspects of the CLVA therapy response of the patients.

The use of novel combination of existing drugs to activate EBV and eliminate virus-infected cells may open the way to more simplified, possibly oral therapies that would greatly benefit patients in developing countries where complex chemoradiation is not possible. Further studies on CLVA are needed for better insight in the molecular basis of tumor or EBV specific immune responses provoked by the lytic induction and the long-term effect of the treatment. The use of EBV as target in therapy could open up new approaches for other EBV-driven tumors.

Acknowledgements

The authors thank the patients who participated in this trial. The authors also thank Max Nobis and Chantal Kuijpers for excellent technical assistance and Renske Fles for organizing the logistics of patient care. The donation of the AGS-BX1 cell line by Dr. L. Hutt-Fletcher is greatly appreciated.

Translational relevance

Undifferentiated nasopharyngeal carcinoma (NPC) is causally associated with Epstein–Barr virus (EBV). As this virus is present in all tumor cells, EBV could serve as a target for therapy. In NPC cells, EBV hides in latency, expressing only a few essential proteins contributing to tumor formation although escaping immune elimination. Inducing the viral lytic phase makes tumor cells susceptible for immune recognition and antiviral therapy. The concept was confirmed in NPC cell lines using gemcitabine combined with a host DNA methylation and histone deacetylation (HDAC) inhibitor as lytic inducer. Subsequent addition of antiviral therapy (ganciclovir) increased specific cytolysis. The cytolytic virus activation (CLVA) therapy was evaluated in 3 patients with progressive end-stage NPC. All patients had stable disease during and for more than 6 months after therapy with improved quality of life. CLVA therapy resulted in increased shedding of viral DNA in the circulation. These data indicate that CLVA therapy may open up a new therapeutic approach for EBV-driven malignancies.
References


