SUSCEPTIBILITY OF HUMAN HERPESVIRUS 8 AS WELL AS OTHER HERPESVIRUSES AND THEIR ENZYMES TOWARDS VARIOUS CHEMOTHERAPEUTICAL AGENTS

Summary of Ph. D. thesis

Gábor Gáspár, MD

Department of Medical Microbiology and Immunobiology
Faculty of Medicine
Albert Szent-Györgyi Medical and Pharmaceutical Centre
University of Szeged
Szeged, Hungary

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INTRODUCTION

Human Herpesvirus 8 and the Family of Herpesviruses

Kaposi's sarcoma (KS), which was firstly described in 1872 by the Hungarian dermatologist Moritz Kaposi, is a mesenchymal tumour involving blood and lymphatic vessels. In 1994 Chang and colleagues identified DNA fragments of a novel gamma herpesvirus, which has been called Kaposi's sarcoma-associated herpesvirus (KSHV), now denominated as human herpesvirus 8 (HHV-8) in a Kaposi's sarcoma skin lesion from a patient with AIDS. HHV-8 sequences have been detected in nearly all forms of KS /both AIDS related and unrelated lesion/, strongly suggesting that this virus plays a crucial role in the pathogenesis of KS, which still remains the most common malignancy associated with HIV infection.

HHV-8 has an ethiological role also in a rare B-cell primary effusion (body cavity based) lymphoma, some cases of multicentric Castleman's disease and possibly in angiosarcoma and angiolymphoid hyperplasia of the skin. HHV-8, the first discovered human Rhadinovirus belongs to the gamma2 group of herpesviruses (rhadinoviruses) together with its simian homologues such as rhesus rhadinovirus (RRV) and Herpesvirus saimiri (HVS) whose natural host is the squirrel monkey. HVS can transform human T lymphocytes.

Among human herpesviruses HHV-8 has the greatest homology to Epstein-Barr virus (EBV), which is a member of gamma1 class herpesviruses (lymphocryptoviruses). HHV-8 resembles Epstein-Barr virus in its tropism for B cells, in its capacity to exist in a latent state, and in the difficulty of replicating the virus for extended periods of time in culture. A productive infection of EBV results in an acute infectious mononucleosis, while the non-productive form is associated with malignancies such as Burkitt's lymphoma, nasopharyngeal carcinomas, and in immunodeficient patients, virus-induced lymphoproliferative disease.

Enzymes of the nucleotide metabolism in herpesviruses

The HHV-8 genome encodes several enzymes that are involved in nucleoside and nucleotide biosynthesis. These include a thymidine kinase (TK), a ribonucleotide reductase (RR), a dihydrofolate reductase (DHFR) and a thymidylate synthase (TS).

Two key enzymes of the nucleotide metabolism are encoded by certain herpesviruses in general and by HHV-8 in particular: thymidylate synthase and dihydrofolate reductase.

Thymidylate synthase is an enzyme that catalyzes the reaction of 5,10-methylenetetrahydrofolate and dUMP to dihydrofolate and dTMP. Since it is the sole *de novo* pathway for the synthesis of dTTP, in the absence of the additional formation of dTMP via thymidine kinase (salvage pathway), the reaction catalysed by TS is the rate-limiting step in DNA synthesis. Therefore, inhibition of TS would prevent cell multiplication. Certain types of tumor cells exhibit increased activity of TS, which is a main target for antitumoral drugs.

VZV has been previously reported to encode a functional TS. HVS contains also a TS gene. Human cytomegalovirus, a virus that belongs to betaherpesviruses does not encode a TS but markedly stimulates cellular TS activity in infected human embryonic lung cells. HHV-8 encodes a TS gene, and protein appeared to be expressed in cytoplasm of cells transfected with an expression vector carrying the HHV-8 TS gene, although no proof of functional enzymatic activity was given.

Dihydrofolate reductase is an ubiquitous enzyme that catalyzes the NADPH-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate which is essential for *de novo* purine, glycine and methionine synthesis and for the biosynthesis of thymidylate.

DHFR is a known target for drug action. Inhibitors of DHFR have proved useful in the treatment of cancer such as methotrexate (MTX) and aminopterin (AMT). DHFR inhibitors such as trimethoprim (TMP) and pyrimethamine (PYR) are potent antibiotics or antiparasitics.

Among herpesviruses, human herpesvirus 8, herpesvirus saimiri, herpesvirus ateles and rhesus rhadinovirus have been found to encode their own DHFR.

Human cytomegalovirus and murine cytomegalovirus do not encode a DHFR but markedly stimulate cellular DHFR activity in quiescent cells. EBV, that does not encode a DHFR, induces a relatively constant level of DHFR mRNA in virus-transformed cells throughout all phases of the cell cycle.

Antiviral strategies against HHV-8 and EBV

HHV-8 replication can be selectively inhibited by several anti-herpesvirus agents including ganciclovir and cidofovir. The incidence of KS was shown to be lower in AIDS patients that had received treatment with ganciclovir. Cidofovir, a broad-spectrum acyclic nucleoside phosphonate analog that does not depend on virus-encoded kinases to become antivirally

active was administered to patients suffering from KS. After five weekly injections of the drug, no clinical, histological, immunohistological, or virological changes could be detected in comparison with saline-injected lesions.

With epidemiological evidence supporting a viral aetiology for KS, interferon became a logical therapeutic candidate. Many trials of interferon have been conducted. In summary, these studies documented a dose-dependent tumor reduction. HHV-8, however, rules over an escape mechanism against interferon due to inhibition of its signalling pathway.

Following primary infection by EBV, the host cell-mediated immune response plays a major role in controlling the lifelong latent persistence of the virus in resting memory B cells. In EBV-seropositive immunocompetent individuals, the number of circulating infected B cells remains low. Patients with congenital or acquired immunodeficiency are susceptible to EBV-associated lymphoproliferations. At least 90% of the posttransplant lymphoproliferative disorders are EBV-associated and can be characterized by a significantly increased EBV load at the time of their diagnosis.

AIMS OF THE STUDY

1. It was well known that HHV-8, RRV and HVS contain a TS gene. We investigated if these gene sequences encode a functional enzyme, and, if so what kinetics can be featured.

Several inhibitors are able to bind either at the pyrimidine substrate or at the folate cofactor sites of TS. We wanted to explore the efficacy of dUMP analogues regarding the inhibition of HHV-8, HVS and VZV TSs.

2. HVS as well as RRV carry a DHFR gene in their genome, but functional activity has so far not been demonstrated for these enzymes. Our research goal was to examine whether these viruses encode a functional enzyme and what characteristics they follow, including their sensitivity to certain inhibitors as well.

3. As far as the evaluation of various antiviral agents concerned, another aim of our investigation was to elaborate a fast, accurate and convenient quantitative real-time PCR

(TaqMan) method to allow the assessment of antiviral activity of molecules against EBV and HHV-8.

MATERIALS AND METHODS

Induction of cell lines

The effects of a selection of anti-herpesvirus nucleoside analogues on the replication of HHV-8 as well as EBV were determined in the producer cell line BCBL-1 and P3HR-1, respectively. Cells were grown in RPMI 1640 medium containing 10% inactivated FBS and antibiotics. Both cell lines were induced by 12-O-tetradecanoyl-phorbol-13-acetate. Cultures were incubated with dilution series of the different drugs for 7 days after which total cellular DNA was extracted.

Isolation of viral DNA

HHV-8 DNA was isolated from the HHV-8 containing BC-3 and BCBL-1 cell line as well as from biopsies of KS lesions obtained from a Belgian as well as from a Hungarian case of classic Kaposi's sarcoma. VZV (strain OKA) DNA was isolated from fibroblasts infected with the virus. DNA was extracted using QIAamp DNA Blood Mini Kit in each case. HVS and RRV DNA were generous gifts.

Enzyme purification

The respective genes were amplified by PCR. The resulting amplicons were cloned in pCR4-TOPO TA vector and the cloned fragments sequenced on both strands. The HHV-8 BC-3 and BCBL-1, HVS, VZV and RRV sequences proved to be 100% identical to the protein sequences available from the National Center for Biotechnology Information Blast GenBank. DNA isolated from the Belgian patient carried an A-G substition at nt position 21047 and at nt position 20824. DNA isolated from the Hungarian patient carried a C-T substition at nt position 21012, an A-G substition at nt position 20776 and a T-A substition at nt position 20177 (positions refer to the numbering used in GenBank U75698). The genes were subcloned in the bacterial expression vector pGEX4. The resulting plasmids were then used to transform competent *Escherichia coli* BL21 cells. Cultures were induced with 0,1 mM

isopropyl beta-D-thiogalactoside for 6 hours at 25 °C. Cells were pelleted at 10000xg for 10 min at 4 °C and resuspended. Nuclease was added for 20 min at room temperature. Clarified lysates were pelleted for 20 min at 15000xg at 4 °C and incubated with Gluthatione Sepharose 4B. The recombinant enzymes were eluted with gluthatione. The protein concentrations were determined and SDS-PAGE was carried out to control the size and purity of the expressed protein.

TS activity assay

The appropriate amount of enzyme was incubated with [5-^{3}H] dUMP in 50 mM Tris-HCl buffer (pH 7.5) containing 5.0 mM formaldehyde, 15 mM beta-mercaptoethanol, 0.1 mM NaF and 5,10-methylene-5,6,7,8-tetrahydrofolate. According to the particular experimental conditions either varying concentrations of dUMP, 5,10-methylene-5,6,7,8-tetrahydrofolate or the respective inhibitors were used. The enzymatic reactions were initiated by addition of the enzyme and were then incubated in triplicates at 37 °C for the appropriate time. The enzymatic reaction was stopped on ice, and 1 ml of active carbon (100 mg/ml in 2% TCA) was added to the tubes, which was thereafter pelleted by centrifugation at 3000 rpm for 10 minutes (4 °C). Tritium release was measured by determining radioactivity. Enzyme kinetics was calculated using Graphpad Prism version 3.02 for Windows.

DHFR activity assay

The assay mixture contained 50 μM DHF, 60 μM NADPH, and 14 μM β-mercaptoethanol in a buffer with pH 7.8 containing 50 mM 2-morpholinoethanesulfonic acid, 25 mM Tris, 25 mM ethanolamine and 100 mM NaCl. The reaction was carried out at 25 °C. The enzyme was added to initiate the reaction. The kinetic decrease in absorbance at 340 nm was measured by a Spectramax 190 plate reader. Varying concentrations of substrates as well as inhibitors were used for the determination of K_m and K_i values, respectively. Enzyme kinetics was calculated using Graphpad Prism version 3.02 for Windows.

Blot hybridisation assay

10 μg of denaturated total cellular DNA of drug-treated producer cell lines and control cells were blotted onto a nylon membrane and UV-cross-linked, after which prehybridization was

carried out for 1 h at 42 ^{0}C. The probe was labelled with digoxenin-dUTP under appropriate PCR conditions. The probe was gel purified, and hybridisation was carried out for 18 h at 42 ^{0}C with 30 ng of the digoxigenin-11-dUTP labelled probe per ml. The membrane was washed at high stringency for 10 min at room temperature followed by two washes of 15 min each in 0.1x SSC-0.1% sodium dodecyl sulfate at 65 ^{0}C. After incubation in blocking buffer, the filter was incubated with an anti-digoxigenin antibody and conjugated with alkaline phosphatase and detection of chemiluminescence was performed by standard methods.

Quantitative PCR assay

Real time detection based on the TaqMan technology was established for both viruses. The PCR primers for EBV detection were designed based on the BNRF1 gene. The forward primer was 5'-CGGCCGTGATGGAGGCTATG-3', the reverse primer was 5'-AGACAGAGGCCACCACGG-3', and the TaqMan probe, which was labelled with the reporter dye 6-carboxyfluorescein (FAM) at the 5' end and the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3' end, was 5'-TGACCTTTGGCTCGGCCTCCTGC-3'. PCR primers and the fluorogenic probe used for HHV-8 quantification were generated based on the ORF73 gene sequence. The forward and reverse primer sequences were 5'-CCGAGGACGAAATGGAAGTG-3' and 5'-GGTGATGTTCTGAGTACATAGCGG-3', and the probe sequence was 5'-FAM-ACAAATTGCCAGTAGCCCACCAGGAGA-TAMRA -3'. The PCR reactions were performed in 96-well optical reaction plates with final volumes of 25 µl per well. The TaqMan assay was performed using an ABI Prism 7700 Sequence Detection System. The PCR mixture contained 12.5 µl of TaqMan universal PCR master mix, 300 nM of each primer, 100 nM TaqMan probe and 2.5 µl template DNA. The PCR conditions for this assay were: 2 min at 50 °C, then 10 min at 95°C, followed by 55 cycles 15 s at 95°C and 1 min at 60°C each. All assays included two negative controls and a dilution series of the plasmid standard. The standard curve of the threshold cycle (C_T) values was constructed for each PCR assay. All PCR measurements were performed in duplicate.

RESULTS

Thymidylate synthase

We cloned and expressed TSs of HHV-8, RRV, HVS and VZV. We also demonstrated that the HHV-8 TS genes derived from the HHV-8 positive BC-3 lymphoma cell line as well as from two HHV-8 positive KS biopsies (one from a Belgian and one from a Hungarian patient) encode enzymatically active proteins. In addition, recombinant TSs derived from HVS as well as from VZV exhibited TS activity. We were, however, not able to show functional TS activity to be associated with the RRV enzyme even though alternative cloning strategies were used and a protein of the correct size was expressed. The question remains whether the RRV TS is indeed functionally active or whether the particular RRV strain that was used here, encodes a defective TS. We next studied and compared the particular kinetics of the TS of HHV-8, HVS and VZV. K_m values with both substrates (dUMP and CH_2H_4-folate) and k_{cat} values for the three herpesvirus enzymes were very comparable and were in the same range as the values reported for human TS. We next studied the inhibitory effect of three well-known TS inhibitors: 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), 5-iodo-2'-deoxyuridine-5'-mono-phosphate (IdUMP), (E)-5-(2-bromovinyl)-2'-deoxyuridine-monophosphate (BVdUMP) and the product inhibitor dTMP on the different viral TS. The type of inhibition was competitive with respect to dUMP for all three analogues studied. The TS encoded by HHV-8, HVS and VZV proved equally susceptible to the compounds as the human, murine and lactobacillus TSs. The HHV-8-encoded TS may not be therefore a target for antiviral therapy.

Dihydrofolate reductase

We cloned and overexpressed the DHFR of HHV-8, RRV and HVS in *E. coli*. Recombinant proteins that were obtained from these three sources all proved enzymatically active. The particular kinetics of the DHFR of HHV-8, HVS and RRV were investigated. K_m values with DHF and NADPH as a substrate for the DHFRs encoded by HHV-8, HVS and RRV as well

as their k_{cat} values proved very comparable for the three herpesvirus enzymes. The K_m value for DHF of the three viral enzymes was invariably 5-15-fold higher than that of the human counterpart, whereas the K_m values for NADPH as a co-enzyme were similar to that of the human enzyme. The k_{cat} values for the three herpesviruses were 1,5-2 fold less than the turnover number characteristic for the human DHFR. We next studied the inhibitory effect of five antifolates, i.e.: MTX, AMT, TMP, PYR and N(α)-(4-amino-4-deoxypteroyl)-N(δ)-hemiphtaloyl-L-ornithine (PT523) on the different viral DHFRs. The three viral DHFRs behaved similarly in their sensitivity to these inhibitors whereby the order of activity ranked as follows: TMP<<PYR<AMT<MTX<PT523. The viral DHFRs, however, proved markedly less susceptible to these inhibitors than the human enzyme. This may indicate that the herpesvirus DHFR is not a good target for selective antiviral therapy.

Antiviral activity of molecules against HHV-8 and EBV

As for HHV-8, ganciclovir, cidofovir and brivudin proved nearly equipotent in their antiviral activity by the quantitative PCR (Q-PCR) method (with EC_{50} values of 1-2 μg/ml). These values are in the same range, if not almost identical, to the data as obtained by the hybridisation assay. Adefovir and acyclovir were somewhat less active; also for these compounds the EC_{50} values generated by both methods were very similar.

The effects of the compounds in focus were assessed on the replication of EBV in P3HR1 cells. Cidofovir proved to be the most potent as an inhibitor of EBV replication. Adefovir and ganciclovir exhibited equipotent activity that was about 3-10-fold more pronounced than that of acyclovir. Brivudin conferred the weakest antiviral activity. Also for EBV, the EC_{50} values, as obtained by the Q-PCR method, proved very similar to the values obtained by the hybridisation assay.

DISCUSSION

Thymidylate synthase and dihydrofolate reductase

The genes for HHV-8 DHFR and TS (ORF 2 and 70, respectively) belong to the group of early viral genes. They are located in the cytokine-containing segment of the HHV-8 genome and not in locations corresponding to HVS ORFs 2 and 70. On the other hand, the location of

the RRV DHFR gene corresponds with that of the HVS DHFR gene. HVS DHFR has a distinct origin and has undergone much less substitution than the HHV-8 DHFR. HHV-8 DHFR protein is much farther diverged from the human DHFR than is the HVS version, implying that they were probably acquired as host cell cDNAs by independent evolutionary events. The HVS DHFR gene is located behind the saimiri transforming protein (STP) which acts as an oncogenic protein and the RRV DHFR is located behind the R1 protein which is also endowed with oncogenic properties. In contrast, the HHV-8 DHFR is located from the oncogenic K1 gene nine reading frames downstream. The fact that these viruses acquired both enzymes of the TS/DHFR complex may be indicative for an important role in providing the infected cell with sufficient thymidylate for viral DNA synthesis. It remains unclear, however, why certain herpesviruses encode their own TS and DHFR, whereas other herpesviruses do not. Possibly, DHFR and TS are involved in oncogenesis and play a role in providing the transformed cell with sufficient quantities of thymidylate. The fact that VZV, which is not an oncogenic herpesvirus, encodes a functional TS, does not necessarily argue against this hypothesis since VZV does not encode the accompanying DHFR and VZV TS is even not essential for replication in vitro. The gammaherpesvirus-encoded TS and DHFR may increase viral replication efficiency and may play a role during persistent viral replication in resting cells, such as lymphocytes, that contain low levels of cellular DHFR.

Antiviral action against HHV-8 and EBV

Antiviral strategies against HHV-8 and EBV can challenge various research pathways involving direct investigation of tumour cells and utilization of an animal virus model system as well; however, real time quantitative PCR evaluates the antiviral activity of molecules against HHV-8 and EBV in a rapid but convenient and accurate fashion. The antiviral features of the reference molecules acyclovir, ganciclovir, cidofovir, adefovir and brivudin, as assessed by this methodology, resembled to a large extent the activity profile determined by DNA-DNA hybridisation method.

CONCLUSION

1. We demonstrated that HHV-8, obtained from the lymphoma cell line BC-3 as well as from Kaposi's sarcoma lesions, carries a gene that encodes a functional thymidylate synthase. The particular characteristics of this enzyme were described and compared to the characteristics of TSs encoded by other organisms.

2. We overexpressed and purified from *E. coli* the dihydrofolate reductases of HHV-8, HVS and RRV. All three enzymes proved catalytically active. The detailed characteristics of these enzymes were determined and compared to each other and their human counterpart.

3. A real time quantitative PCR was developed to assess antiviral activity of molecules against HHV-8 and EBV. The antiviral activity of acyclovir, ganciclovir, cidofovir, adefovir and brivudin, as assessed by this methodology, proved very similar to the activity as determined by a DNA-DNA hybridisation method.

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