Original article

Retinoic acid analogues inhibit human herpesvirus 8 replication

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Background: Retinoids have a pronounced antiviral effect against several viruses. In this study we aimed to investigate the effect of retinoids on human herpesvirus 8 (HHV-8).
Methods: A panel of retinoic acid compounds were tested for their antiviral activity against HHV-8 in human umbilical vascular endothelial cells (HUVECs) and in a human epithelial cell line. The presence, transcription and antigen expression of HHV-8 in infected cells – in the presence or absence of retinoic acid compounds – were evaluated by PCR, reverse transcriptase PCR and immunofluorescence assays; HHV-8 viral load was determined by real-time quantitative PCR. Angiogenesis induced by HHV-8 was also assessed using Cultrex® basement membrane extract.
Results: The compounds tested specifically inhibited viral promoters, during the early and late phases of infection in both cell systems tested, and resulted in up to 100-fold reduction of viral titre and release of progeny virus. The inhibition of viral replication induced by retinoids in endothelial cells, the primary target of HHV-8-driven transformation in Kaposi's Sarcoma, prevented endothelial cells from developing spindle morphology and *in vitro* tube formation, characteristic changes associated with HHV-8 infection and transformation.
Conclusions: We show that retinoids inhibit HHV-8 replication and identify new retinoid compounds with a strong antiviral effect. Selective retinoids, particularly those with retinoic acid receptor agonist activity, may be good candidates for the development of antiviral drugs.

Introduction

Human herpesvirus 8 (HHV-8) is the most recently discovered human herpesvirus. Similarly to all other herpesviruses, after primary infection HHV-8 persists in the infected host in a latent state and the virus can switch from latent to lytic replication, mainly when the immune competence of the host is altered. Viral sequences are detected in the healthy population at variable rates, depending on the geographical area, and prevalence values >50% are common in tropical regions. However, the clinical syndrome associated with primary infection is poorly defined, and only neoplastic diseases have been so far specifically linked to HHV-8 reactivation. In addition to Kaposi's Sarcoma (KS), HHV-8 is associated with lymphoproliferative diseases such as primary effusion lymphoma (PEL) and multicentric Castelman's Disease (MCD) (reviewed in [1]).

KS is a multifocal proliferative lesion that includes different subtypes. Classic KS is an indolent skin tumour occurring mainly in elderly men of Mediterranean origin. The endemic form of KS affects younger individuals in sub-Saharan Africa and has a more aggressive course. Iatrogenic KS occurs in transplant recipients and usually regresses on removal of immunosuppressive therapy. KS is the most common neoplasia in AIDS patients and in this case the disease

is very aggressive and often displays mucosal progression, with visceral dissemination and high mortality. Although these types of KS have different epidemiology and morbidity, they are histologically indistinguishable. All KS lesions are characterized by a florid angiogenesis with the formation of a dense capillary network and contain a variety of cell types, including infiltrating inflammatory cells, extravasated erythrocytes, and the characteristic KS spindle cells [2,3]. The KS spindle cells are a heterogeneous cell population of mesenchymal origin expressing both endothelial and macrophage markers. HHV-8 has a major role in the development of all forms of KS. In fact, the virus is present in all KS lesions and HHV-8 active replication precedes the onset of KS and denotes high risk of progression to KS [4]. Furthermore, HHV-8 infection causes *in vitro* transformation of microvascular endothelial cells [5]. Even if virus infection is necessary for the development of KS, it is not sufficient, as only a minority of HHV-8-infected individuals will develop the disease. KS preferentially arises in a context of immune dysfunction, such as in HIV-infected patients or transplant recipients, while the classic and endemic forms occur in a context of immunosenescence or conditions that interfere with the immune system.

The mechanisms underlying HHV-8-dependent transformation are complex and are probably due to the coordinated expression of several viral functions. HHV-8 encodes several genes that inhibit apoptosis (ORF73/LANA, ORF16/vBcl-2, K7, vIRF-1, vIRF-2, K10.5 and vFLIP), control the cell cycle (ORF73/LANA and ORF72/V-cyc), and directly drive transformation of infected cells (K12/kaposin, K1/VIP and ORF74/vGCR) (reviewed in [1]). Furthermore, the viral G-protein-coupled receptor induces the expression of vascular endothelial growth factor, a cytokine that is important in KS-associated angiogenesis. However, the full extent of the concerted action of these genes has still to be analysed in detail.

Antiviral compounds efficient to control viral infection are lacking. *In vitro* studies have shown that some of the currently available antiherpetic drugs, including foscarnet, cidofovir and ganciclovir, inhibit HHV-8 replication [6,7]. However, the effect of antiherpetic drugs *in vivo* is controversial. Some reports claim efficacy, but other reports describe poor therapeutic effects of antiherpes drugs in HHV-8-related disorders [8]. The studies on HHV-8 sensitivity to antiviral drugs are hindered by the lack of a cell system supporting exogenous productive infection and by the fact that HHV-8 does not cause detectable cytopathic effects in cell culture.

Retinoic acid (RA) has been proposed for the treatment of KS. RA isomers derive from vitamin A and act after binding to specific nuclear RA receptors (RARα, RARβ and RARγ) and retinoid X receptors (RXRα, RXRβ and RXRγ). Recently, Alitretinoin (9-*cis*-RA, an RAR and RXR pan-agonist) has been approved for the topical treatment of KS in HIV-infected patients [9]. Clinical trials show that topical application of 9-*cis*-RA induces clinical responses in ~40% of patients [10–13]. Oral administration of 9-*cis*-RA produces similar results, but with more pronounced adverse effects owing to toxicity [14–16].

The antineoplastic mechanisms of RA in the context of KS are only partially understood. It is well ascertained that RA has antiproliferative effects on neoplastic cells of various origin, but only a limited number of studies have characterized the action of RA on the proliferation of KS cells. Corbeil *et al.* [17] showed that a panel of RA isomers inhibited the growth rate of cell cultures derived from biopsies of KS lesions. It was also shown that RA inhibits the production of interleukin-6, an autocrine growth factor for KS cells, and that addition of exogenous interleukin-6 to RA-treated KS cultures abrogates the cell growth inhibition [18].

Several studies have shown that RA treatment of KS is associated with inhibition of angiogenesis and a decreased vascular response both *in vitro* and *in vivo*. It has been reported that RA significantly decreases tumour cell induced angiogenesis in mice and rats [19]. All-*trans*-RA makes endothelial cells refractory to migration induced by purified angiogenic factors, without affecting cell proliferation [20]. A recent study showed that the synthetic retinoid fenretinide blocks KS tumour growth through modulation of angiogenesis [21].

Retinoids also have a pronounced antiviral effect against several viruses including HIV, human papillomavirus, hepatitis C virus, and several herpesviruses, such as herpes simplex virus, Epstein-Barr virus (EBV) and cytomegalovirus (reviewed in [22]). However, it has never been determined whether the biological effects induced on KS cells are due only to the antiproliferative activity of retinoids or are also related to an inhibition of HHV-8 replication. In this report we describe productive infection with HHV-8 of human epithelial and endothelial cells, and provide evidence that 9-*cis*-RA, as well as synthetic retinoids, has biologically and therapeutically relevant antiviral effects on HHV-8-infected cells.

Methods

Synthesis of retinoids

Compound UVI2513 (Figure 1A) was prepared by iterative Horner-Wadsworth-Emmons (HWE) reactions [23] starting from the phenanthrene 9-carboxaldehyde. A HWE condensation with triethylphosphonoacetate was followed by diisobutylaluminium (DIBAL) reduction and oxidation of the

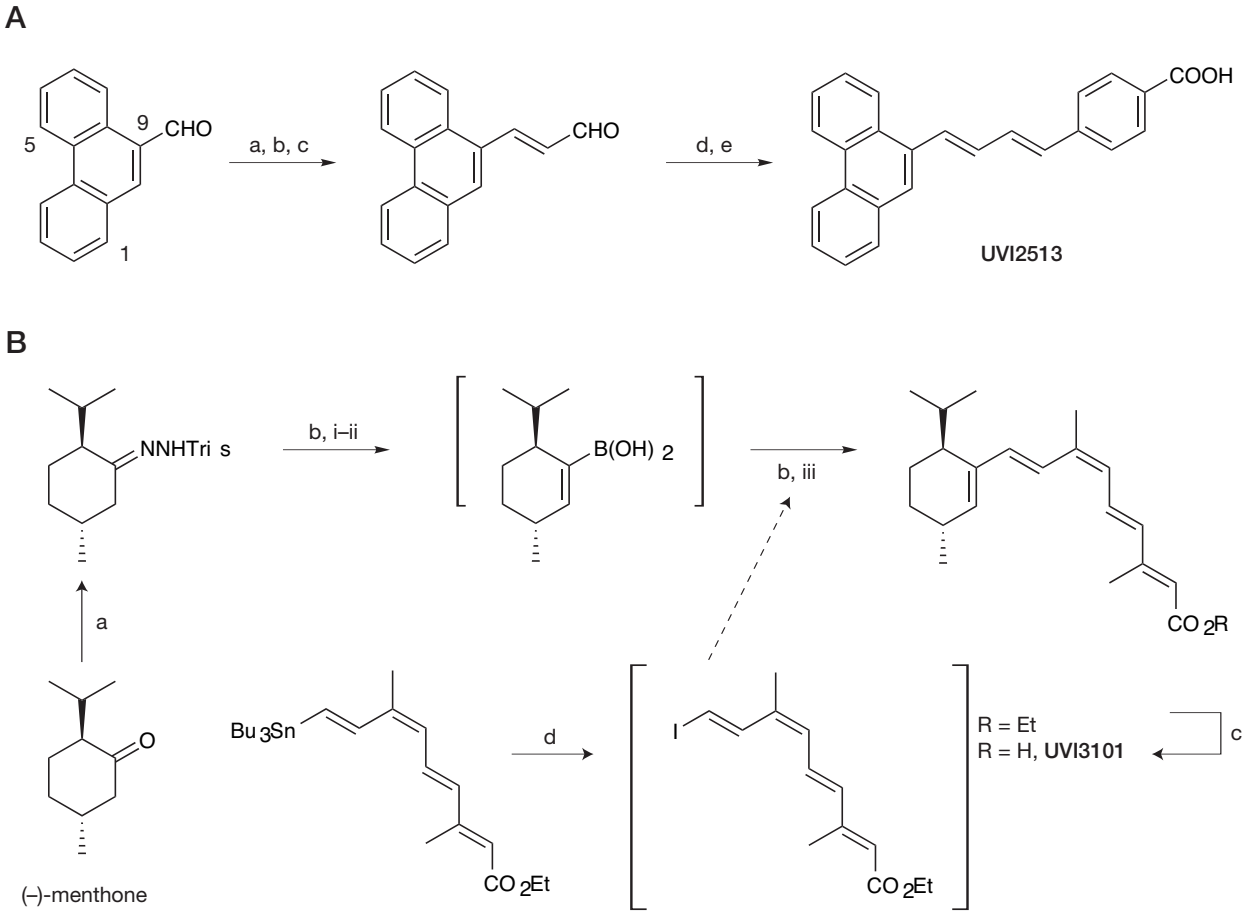
allylic alcohol with activated MnO_2. The second HWE reaction with the benzyl phosphonate afforded the entire skeleton of the arotinoid, which upon saponification provided UVI2513. Yields of the entire sequence are uniformly high (80–99%) and no mixtures of stereoisomers are obtained, as the HWE reactions take place with high E-stereoselectivity and this is maintained along the sequence.

The synthesis of UVI3101 (Figure 1B) was performed following the approach developed in our group [24] based on the C6–C7 disconnection that uses a Suzuki reaction of an alkenyl boronic acid (generated *in situ* from the trisylhydrazone of a cycloalkanone) and a tetraenyl iodide (the C_{11} polyene fragment). Upon hydrogen abstraction and Shapiro reaction, the less-substituted kinetic alkenyllithium reagent was obtained from the trisylhydrazone of (–)-menthone. Quenching of the anion with tri-isopropylborate afforded the corresponding alkenylboronate or boronic acid, which was coupled to the tetraenyliodide using TlOH as promoter, providing the final arotinoid after saponification.

HHV-8 infection

Cell-free HHV-8 virus inocula were obtained from the PEL-derived chronically infected BC-3 cell line and quantitation of virus genomes present in the stock was obtained by real-time PCR, as previously described [25]. Prior to use, virus stock was treated with DNase I and RNase A, to eliminate free viral nucleic acids potentially present in the preparation. The absence of free viral nucleic acid was assessed by sensitive nested PCR. The viral titre was determined by real-time PCR

Figure 1. Synthesis and structure of the retinoids UVI2513 and UVI3101



(A) Synthesis and structure of UVI2513. Reagents and conditions are described: a, $(EtO)_2P(O)CH_2COOEt$, *n*-BuLi, 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU), tetrahydrofuran (THF), -60°C; b, diisobutylaluminium hydride (DIBAL-H), THF, -60°C; c, MnO_2, CH_2Cl_2 room temperature, 7h; d, $(EtO)_2P(O)CH_2C_6H_4COOMe$, *n*-BuLi, DMPU, THF, -60°C; e, 5N NaOH/MeOH (1:2), reflux, 4h. **(B)** Synthesis and structure of UVI3101. Reagents and conditions are described: a, $TrisHNNH_2$, CH_2Cl_2, 0°C, 2 h (91%); b, i) n-BuLi, 4:1 hexane/TMEDA, -78°C, 30 min; ii) $B(OiPr)_3$, -78 to 0°C; iii) $Pd(PPh_3)_4$, tetraenyliodide, 10% aqueous TlOH, 25°C, 15 min (71%); c, 5N KOH/EtOH, 70°C, 30 min (92%); d, I_2, CH_2Cl_2, 25°C.

and all virus inocula used in the experiments were adjusted to contain the same amount of viral DNA. Control cultures were infected with UV-irradiated HHV-8 inoculum and inactivation was confirmed by lack of viral transcription, as assessed by reverse-transcription PCR (RT-PCR). Aliquots of cells and supernatant were collected at regular time intervals to monitor the amount of viral DNA and RNA produced in infected cells and to measure the release of infectious virus in the supernatant.

Infection studies were performed in primary human umbilical vascular endothelial cells (HUVECs), a natural target of *in vivo* infection, and in the human epithelial 293 cell line, which is not a natural target of infection but represents a convenient system for performing serial experiments. HUVECs were isolated from umbilical vein as described [26] and cultured in collagen-coated flasks (Biocoat Collagen, BD Biosciences, Bedford, USA) with endothelial cell basal medium (EBM) complete medium (BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal calf serum (FCS). Second to fifth passage HUVECs were used for all experiments. The 293 cell line (ATCC CRL-1573) was grown in Dulbecco's modified Eagles medium medium supplemented with 10% FCS.

Cell infection was performed as already described [27], always using the same amount of virus inoculum. Control samples were mock infected with UV-inactivated HHV-8, obtained by exposing the purified inoculum under UV light (200 mJ/cm^2) for 30 min.

Retinoids were added to cell cultures at 10^{-6} M concentration. No cytotoxicity was observed in any of the compounds used, as shown by MTT assays, performed in parallel with each experiment, as described [28]. Briefly, MTT assays were performed on triplicate samples of 293 and HUVEC cells incubated with serial dilutions of RA compounds (from 10^{-4} to 10^{-9} M) in 96-well plates (10^4 cells per well in 100 µl). At 1, 3, 7 and 14 days from the beginning of cell treatment, cells were analysed for MTT reduction by adding 1 mg/ml MTT to each well for 4 h and 100 µl/well dimethyl sulfoxide for a further 2 h. Absorbance was finally evaluated by reading plates at an OD of 570 nm. Statistically significant inhibition of cellular reduction activity was observed only in samples treated with 10^{-4} and 10^{-5} M concentrations, and no effect was detected in cultures treated with lower concentrations of RA compounds.

The presence, transcription and antigen expression of HHV-8 in infected cells were evaluated by PCR, RT-PCR and immunofluorescence assays (IFA); HHV-8 viral load was determined by real-time quantitative PCR as already described [25,27].

To analyse the infectivity of released virions, the culture supernatant collected from infected cells was treated with DNAse, filtered through 0.5 µm pore size membranes and used to infect a target culture of uninfected HUVECs. Virus transmission was assessed by PCR and RT-PCR performed on total DNA and RNA extracted from target infected cells.

Interferon testing

The production of interferon (IFN) species by HUVEC cells treated with RA compounds was investigated on cell culture supernatant collected at days 1, 2, 3, 5 and 7. Specific ELISA tests for IFN-α, -β and -γ (Biosource™, Biosource Europe SA, Nivelles, Belgium) were employed following manufacturer's instructions and using positive controls provided in the kits.

Analysis of HHV-8-induced angiogenesis

In vitro capillary-like structure formation assay was performed using the Cultrex® Basement Membrane Extract (BME) (Cultrex®, Trevigen Inc., Gaithersburg, MA, USA), as described [27]. The soluble BME was placed at 50 µl/well in eight-multiwell chamber-slides. The slides were incubated at 37°C for 30 min, then treated or untreated infected and uninfected HUVECs were seeded at 5×10^4 cells/well and incubated at 37°C. Tube formation on BME was analysed using phase-contrast microscopy.

Luciferase assays

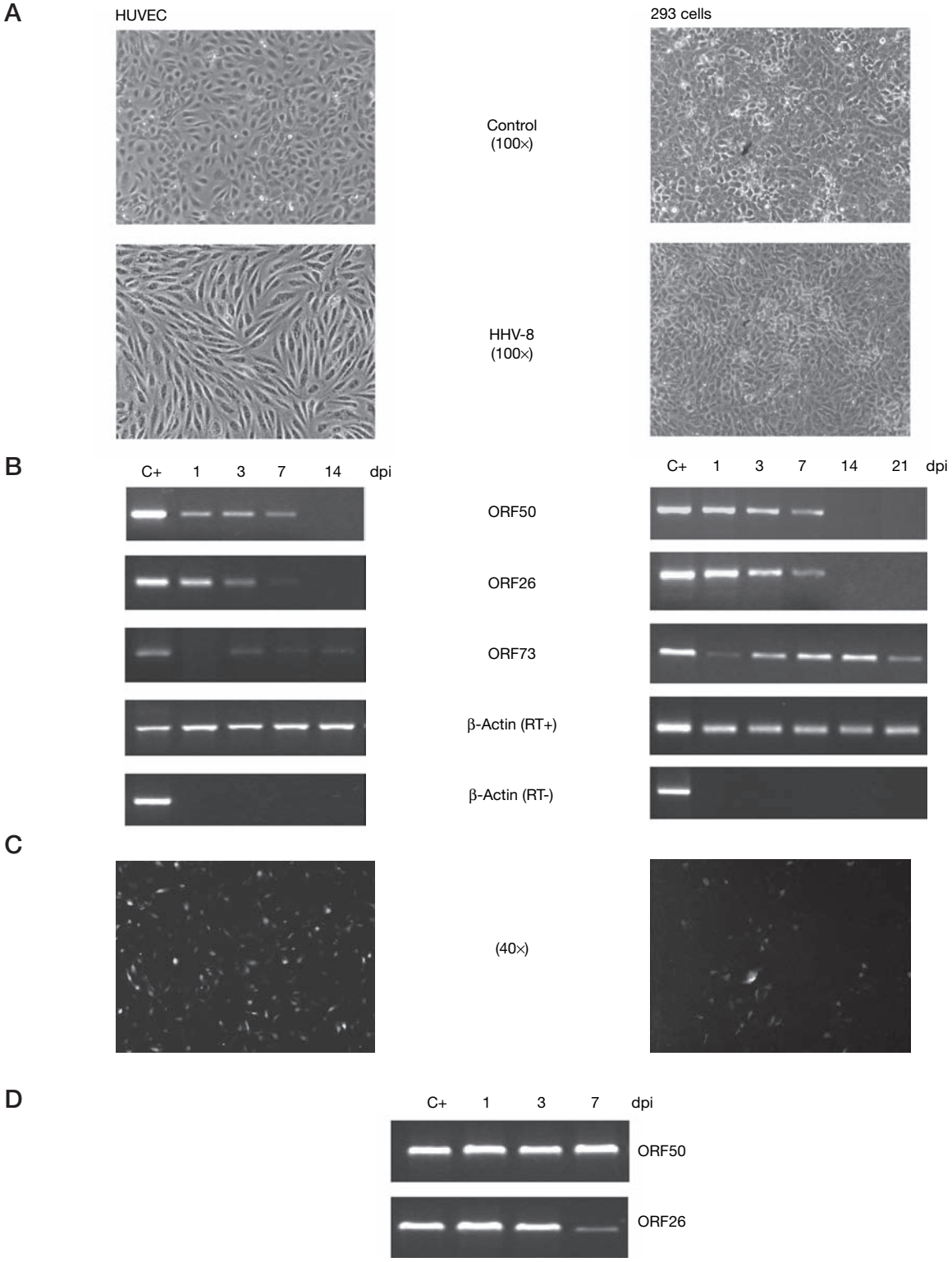
To analyse the effect of RA derivatives upon the transcriptional activation of HHV-8 promoters, 293 cells were cotransfected with a plasmid coding for HHV-8 ORF50 activator (pCR-ORF50) and reporter constructs containing the luciferase gene under the control of different HHV-8 promoters (pGL-ORF57pr, pGL-T1.1pr and pGL-TKpr) [29], in the presence or absence of 10^{-6} M of selected retinoids. Cells collected 48 h post-transfection were analysed by luciferase and β-galactosidase assays. All luciferase activities were normalized to the corresponding β-galactosidase activities. Fold activation was calculated comparing the normalized luciferase activity stimulated by the pCR-ORF50 to that stimulated by the control empty pCR vector with an assigned activity of one.

Results

HHV-8 *in vitro* infection model

HHV-8 infection of HUVEC cells followed the same course as recently described by our group [27]. Infected HUVECs develop a typical spindle-shaped phenotype, characteristic of HHV-8 infection (Figure 2A), and acquire the ability to form tube-like structures when seeded on BME. HHV-8 establishes a productive replication in HUVECs, shown by the presence of viral transcripts from lytic genes (Figure 2B), and by IFA

Figure 2. Characterization of HHV-8 infection in HUVEC and in 293 cells



(A) HUVEC or 293 cell monolayers, mock infected (control) or infected with human herpesvirus 8 (HHV-8), observed 48 h post-infection (pi) with a Nikon Eclipse TE2000-S microscope equipped with phase-contrast (magnification 100×). **(B)** PCR and reverse transcriptase PCR amplification of DNA and RNA extracted from infected HUVECs or from 293 cells. Samples collected at different days pi (dpi) were analysed for the indicated HHV-8 genes and for human β-actin. Amplification of β-actin mRNA with (RT+) and without (RT-) retrotranscription are shown. Positive controls of amplification of the genes analysed (C+) are included. **(C)** Expression of HHV-8 late K8.1 protein at 48 h pi in HHV-8-infected HUVEC or 293 cells. Immunofluorescent micrographs are shown at 40× magnification. Approximately 20% of HUVECs and 5% of 293 cells were positive for HHV-8 K8.1 protein. **(D)** Culture medium of infected HUVECs was filtered with 0.5 μm mesh and used as inoculum for infecting fresh HUVEC cultures. PCR amplification of HHV-8 ORF50 and ORF26 of DNA extracted from HUVEC at different days pi is shown.

Table 1. Anti-HHV-8 activity of retinoid compounds (estimated by semiquantitative PCR on 293 cells)

Compound*	Fold inhibition of HHV-8	RAR and/or RXR modulation	Type of activity and receptor isotype selectivity
UVI3101	100	RAR, RXR	Partial RAR agonist, weak RXR agonist
UVI2513	100	RAR	RARβ-selective agonist with weak RARα agonist activity
LG100754	100	RXR	RXR/RXR homodimer antagonist RXR/RAR heterodimer agonist [43]
9-*cis* Retinoic acid	100	RAR/RXR	Pan-agonist for both RAR and RXR
AGN193109	10	RAR	Inverse pan-agonist [44]
CD666	10	RAR	RARγ selective agonist [45]
ALRT1550	10	RAR	Pan-RAR agonist [46]
BMS453	10	RAR	RARβ agonist and RARα, -γ antagonist [47]
UVI2521	10	RAR	Pan RAR agonist [48]
BMS493	0	RAR	Inverse pan-agonist [47]
LG100567	0	RAR	RAR pan-agonist [46]
UVI2105	0	RXR	RXR pan-agonist [49]
LG1069	0	RXR	RXR pan-agonist [50,51]
UVI2107	0	RXR	RXR pan-agonist [52]
SR11237	0	RXR	RXR pan-agonist [53]
LG101506	0	RXR	RXR pan-antagonist [54]

*HHV-8, human herpesvirirus 8; RAR, retinoic acid receptors; RXR, retinoid X receptors.

with a monoclonal antibody to K8.1, a late viral protein (Figure 2C). The peak of viral replication takes place 3 days post-infection (pi), shown by the percentage of IFA-positive cells (~20%) and by real-time PCR quantitation of viral DNA, which consistently ranged above 2×10^5 copies/μg of cell DNA, corresponding to about 10 copies of viral DNA per IFA-positive cell. The development of productive replication in HUVECs was confirmed by detection of infectious virus released in culture supernatants. In fact, 3×10^4 copies/ml of virus DNA were detected in culture supernatants and treatment of samples with DNAse did not abolish positivity, suggesting that viral sequences were packaged within virion particles. Furthermore, the virions released by HUVECs were infectious, as determined by using filtered supernatants to infect fresh HUVEC cultures. Three and seven days after infection the cultures harboured viral transcripts encoded both by immediate early (ORF50) and late (ORF26) genes, confirming the presence of infectious virus (Figure 2D).

HHV-8-infected 293 cells did not develop any cytopathic effect or morphological change (Figure 2A). PCR and RT-PCR of infected 293 cells showed the presence of viral DNA and mRNA corresponding to lytic viral genes (Figure 2B), suggesting that productive infection took place and that the virus was replicating. The levels of replication were significantly lower than those detected in HUVECs as shown both by the lower number of IFA-positive cells (~5%, Figure 2C) and by real time PCR quantitation of viral DNA, which peaked 7 days pi and reached 10^4 copies/μg of cell DNA. Infectious virus was detected in the culture supernatant, at 5×10^3 copies/ml, as late as 7 days pi.

Effects of retinoids on HHV-8-infected cells.

A panel of retinoids was analysed for antiviral activity on HHV-8-infected 293 cells. The panel included both well-characterized retinoids as well as newly synthesized molecules. The RAR/RXR selectivity (and, in some cases, activity) profile of the compounds are shown in Table 1. The activity profile (RAR agonists, RXR agonists and RXR antagonists) of the new retinoids was determined by Gronemeyer's laboratory at the Institute of Genetics and Molecular and Cellular Biology (IGBMC, Illkirch, Strasbourg; data not shown). The retinoid 9-*cis*-RA was also included in the analysis. Aliquots of cells and supernatant from infected cultures were collected 3, 7 and 14 days pi, and viral replication and release were assessed by semi-quantitative PCR and RT-PCR. All experiments were performed with RA compounds at a concentration of 10^{-6} M a dose at which no toxic effect was detected, as shown by MTT analysis. Cell doubling was not affected, as determined by cell counts, and apoptosis was not induced, as indicated by Annexin V/propidium iodide staining and fluorescence-activated cell sorting analysis (data not shown). Toxicity testing by MTT

analysis was included in all experiments, confirming that at this concentration none of the RA compounds induced toxic effects.

At all time points the majority of retinoids showed a significant antiviral activity, reflected by a 10-fold reduction in the levels of HHV-8 DNA and RNA in infected cells (Table 1), as estimated by semiquantitative PCR and RT-PCR. A similar reduction was also detected in the amount of viral DNA present in the culture medium. Most of the active compounds were RAR agonists, whereas selective RXR agonists showed no effect on HHV-8 replication. Compound UVI2112 and two newly synthesized molecules, compounds UVI3101 and UVI2513 (Table 1), together with 9-*cis*-RA showed a more pronounced effect and produced a 100-fold decrease of viral load and replication. Only a few compounds did not inhibit HHV-8 replication. However, the analysis was carried out by semiquantitative PCR and therefore lower grade inhibition (two- to fivefold) could not be appreciated. The half maximal inhibitory concentration (IC_{90}) of the most active molecules was determined, revealing that RA compounds had a significant activity even at nanomolar concentrations: 9×10^{-9}M for 9-*cis*-RA, 2×10^{-8}M for UVI2513, 5×10^{-8}M for UVI3101 and 2.5×10^{-7}M for UVI2112. This screening was carried out on 293 cells, which were easier to handle and manage than endothelial cells. However, 293 cells do not constitute a natural target of viral infection, even if they do support low levels of *in vitro* viral productive replication. Therefore, the antiviral activity of the most effective compounds (that is, 9-*cis*-RA, UVI3101, UVI2513 and UVI2112) was confirmed on HUVECs. Also in this case, RA inhibited HHV-8 replication, as reflected by the 100-fold decrease of viral DNA and RNA within infected cells and the decrease in viral DNA sequences detected in the culture media. In fact, real-time PCR analysis showed a 73–103-fold decrease of virus production in both infected cells and culture supernatant in the presence of selected retinoids. Infected cells harboured an average of 3.9×10^5 HHV-8 DNA molecules in 10^5 cells, whereas the same amount of cells infected in the presence of the corresponding retinoid contained only 5.3×10^3 (9-*cis*-RA), 4.1×10^3 (UVI3101), 3.7×10^3 (UVI2513) and 4.5×10^3 (UVI2112) HHV-8 DNA molecules. The inhibition was also confirmed by immunofluorescence analysis, which showed positivity in <1% of cells compared with >20% in untreated infected cells. More interestingly, HUVECs infected in the presence of RA failed to develop the spindle cell morphology associated with HHV-8 replication (Figure 3A), and partly lost the ability to form tube-like structures on matrigel (Figure 3B).

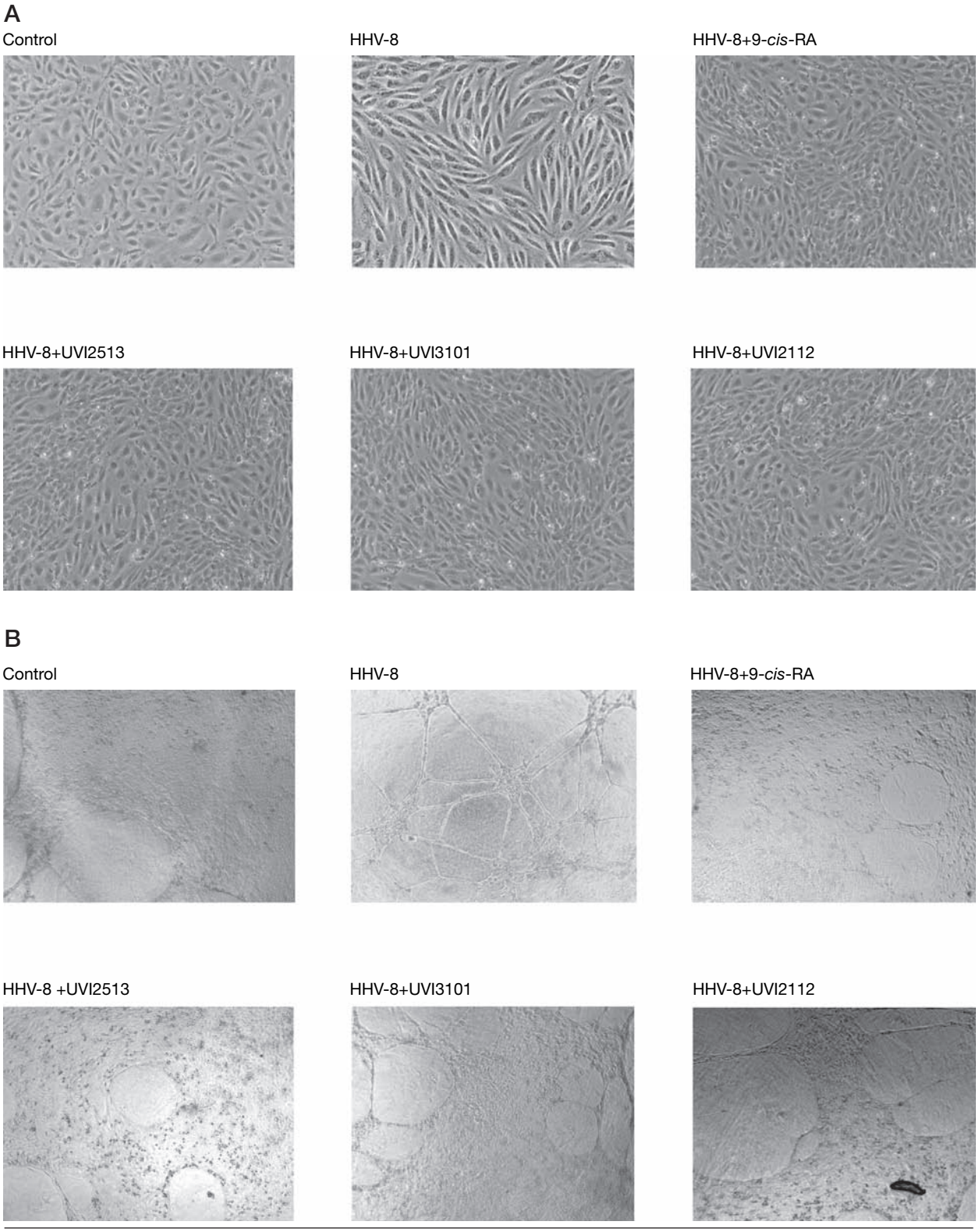
The antiviral effect of RA is generally associated with induction of IFN or with a downmodulation of viral transcription (reviewed in [22]). To investigate the possibility that retinoids induced IFN synthesis, supernatants of 293 and HUVECs cultured for 48 h in the presence of UVI3101, UVI2513 or UVI2112 were analysed by ELISA for the presence of IFN-α and IFN-β. However, no basal production was detected and untreated and retinoid-treated cultures showed no difference (data not shown).

To determine whether retinoids affected viral transcription, 293 cells cultured in the presence of retinoids were co-transfected with a plasmid expressing HHV-8 ORF50 and reporter constructs expressing the luciferase gene under the control of different viral promoters. HHV-8 ORF50 is a potent transactivator, expressed in the immediate-early phase of infection, which activates all viral promoters and is responsible for switching on viral replication. All retinoids significantly affected viral transcription, but different compounds seem to have specific actions (Figure 4): the promoter of ORF57, a transactivating gene expressed during the early phase, is more sensitive to UVI2513; the promoter of T1.1, a gene expressed at high levels during productive replication, is downregulated mostly by UVI2112; and the promoter of TK, a gene involved in DNA replication, is affected mostly by UVI3101.

Discussion

The efficacy of antiviral drugs on HHV-8 replication is difficult to assess, owing to the lack of an efficient *in vitro* system supporting HHV-8 replication and because the virus does not induce evident cytopathic effects. This difficulty is usually partially circumvented by analyzing viral reactivation in HHV-8 chronically infected PEL cell lines. Chemical activation results in a 10-fold increase of viral DNA levels and antiviral activity can therefore be measured by hybridization assays [30] or by real-time PCR quantitation [31,32]. However, this approach is severely limited by the low amount of viral reactivation and by the fact that cells are already infected. Therefore, it is not possible to determine whether the antiviral activity is efficient in preventing infection of new cells and in abolishing the biological effects induced by viral infection. In this report, we describe that a cell-free preparation of HHV-8 inoculum establishes a limited *in vitro* productive infection even in cells that are not natural targets of viral infection, such as the human 293 cell line. We provide evidence to indicate that the system is suitable to assess the efficacy of antiviral compounds. In particular, we show that some retinoids have a significant effect on lytic HHV-8 replication, producing up to 100-fold reduction of viral titre. Furthermore, the antiviral effect was confirmed in endothelial cells, natural targets of HHV-8 infection, confirming that

Figure 3. Retinoids inhibit HHV-8-induced spindle morphology and capillary-like structure formation



(A) Retinoids inhibit human herpesvirus 8 (HHV-8)-induced spindle morphology in HUVECs. Cells mock infected (control) or infected with HHV-8 in the absence (HHV-8) or presence of retinoids (HHV-8+9-*cis*-RA, +UVI2513, +UVI3101 or +UVI2112) were photographed 48 h post-infection (pi). **(B)** Retinoids inhibit HHV-8-induced capillary-like structure formation. Mock infected (control) and HHV-8-infected HUVECs in the absence (HHV-8) or presence of retinoids (HHV-8+9-*cis*-RA, +UVI3101, +UVI2112 or +UVI2513) were harvested 24 h pi and plated on basement membrane extract. Cells were observed after 24 h. The formation of tube-like structures was highly enhanced by HHV-8 infection and was remarkably reduced in the presence of selected retinoids.

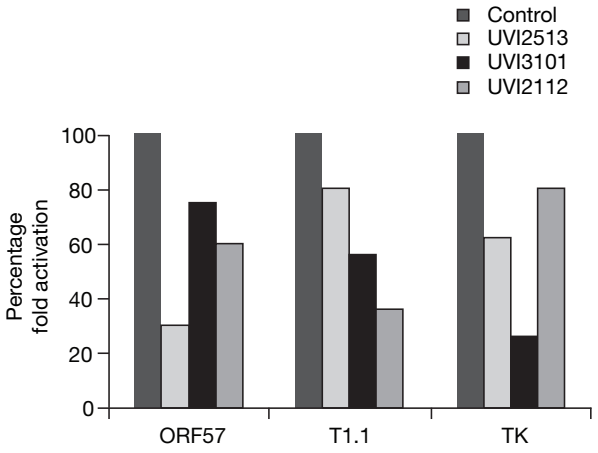
HHV-8 replication is inhibited and indicating that the antiviral effect of retinoids also results in the abolition of HHV-8 angiogenic potential. Therefore, the clinical observation that 9-*cis*-RA is effective in the topical treatment of KS is explained, at least in part, by the direct antiviral action exerted by 9-*cis*-RA on HHV-8.

The antiviral actions of retinoids on several different viruses, including papillomavirus, hepatitis C virus, HIV, EBV and herpes simplex virus, is well documented both by *in vitro* and clinical studies [22]. It is not possible to highlight a common mechanism of antiviral activity, owing to the multiple targets of retinoid action and because of the different biological effects associated with the different classes of retinoids [22]. However, available evidence identifies the existence of two main pathways. One is induction of interferons, resulting in the establishment of a state of resistance to infection [33,34], and the other is inhibition of transcription, resulting in a decreased efficiency of viral replication [35,36]. We show that the active retinoids investigated are able to significantly inhibit HHV-8 transcription, without inducing IFN, suggesting that the observed antiviral effect is at least partially due to retinoid-dependent inhibition of transcription.

Retinoids exert their effects by binding to specific nuclear receptors, which function as transcription factors and regulate the expression of target genes [37]. Two classes of retinoid receptors have been identified, the RARs and the RXRs. It is interesting to note that all the compounds with higher anti-HHV-8 activity are endowed with RAR-agonistic potential, despite the fact that some have also been characterized as RXR ligands (UVI3101 is a pan-RAR/RXR agonist, whereas UVI2112 is an RXR antagonist). In contrast, the majority of retinoids without significant antiviral activity are apparently pure RXR agonists. These findings are consistent with a relevant role of RARs in mediating the inhibitory effects on HHV-8 replication. Additional studies are required to elucidate whether these effects are mediated by all three RAR subtypes. In fact, none of the most potent analogues shows subtype selectivity, and the RARγ-selective UVI2111 (CD666) has lower potency than the leading compounds. Strikingly, UVI-2109 is also potent, despite being an RAR-inverse agonist, but another type of arotinoid with the same profile (UVI2024-BMS493) is inactive. The possibility that distinct RARs may mediate the antiviral activity of RA is of potential clinical relevance, permitting the use of selective synthetic agonists that are probably less toxic compared with pan-RAR/RXR agonists such as 9-*cis*-RA.

Only limited information is currently available on the involvement of distinct RAR and/or RXR subtypes in retinoid-dependent inhibition of viral replication. The most relevant body of data regards the effects of RA on

Figure 4. Activation of HHV-8 promoters in the presence of retinoids in 293 cells



Cells were cotransfected with luciferase reporter plasmids driven by human herpesvirus 8 (HHV-8) promoters (ORF57, T1.1 and TK) and with a control plasmid vector expressing HHV-8 ORF50. The cells were harvested 48 h post-transfection and luciferase activity measured. Values are plotted as percentage fold activation. The results are the average of duplicate samples in three independent experiments.

the EBV lytic cycle. In this setting, it has been shown that RA-dependent inhibition of virus replication is at least partially dependent on transcriptional effects. In fact, RA is able to interfere with the function of the BZLF1 protein, an immediate early gene product that acts as a transcription factor, inducing the switch between the latent and lytic cycle. These effects are related to the ability of both RARα and RXRα to bind a typical response element in the promoter of the BMRF1 gene, a transcriptional target of BZLF1. The control of HHV-8 replication is similar to that of EBV and transcriptional activators and targets of the two viruses share significant homology and conservation of functions [29]. It will be interesting to assess whether retinoids use similar mechanisms to inhibit the HHV-8 lytic cycle. It is relevant to note that active HHV-8 replication is not necessary for maintaining the KS lesion. In fact, most KS cells express only HHV-8 transcripts that are primarily associated with latency, such as LANA, v-FLIP and kaposin [38]. Latent HHV-8 infection is not necessarily oncogenic and only a small minority of individuals latently infected with HHV-8 will develop KS. Instead, reactivation and active HHV-8 replication are prerequisites for KS development [39,40]. This is the reason why, even if KS is associated with HHV-8, the use of antiviral agents does not have a significant effect on the tumour. However, these agents decrease the risk of developing KS in HIV-infected patients [41]. It has also been reported that RA does not control the original tumour, but can block the development of new primary tumours [42]. Therefore, it

is possible that the success of RA in the treatment of HIV-associated KS is not limited to a direct effect on tumour cell biology, but instead RA probably targets viral functions triggering cell transformation and neoplastic progression. On these grounds, the results of the present study stimulate further research to elucidate the effects of retinoids on virus-related activities relevant for cell transformation and strengthen the rationale for the use of retinoids in the prevention and treatment of HHV-8-associated disorders.

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