

ORIGINAL ARTICLE

Antimicrobial sulfonamides clear latent Kaposi sarcoma herpesvirus infection and impair MDM2–p53 complex formation

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Kaposi sarcoma herpesvirus (KSHV), also known as human herpesvirus 8, is the causative agent of Kaposi sarcoma; this malignant angiosarcoma is usually treated with conventional antitumor agents that can control disease evolution, but do not clear the latent KSHV episome that binds to cellular DNA. Some commercial antibacterial sulfonamides were tested for the ability to suppress latent KSHV. Quantitative PCR (qPCR) and cytofluorometry assays were used for detecting both viral DNA and the latency factor LANA (latency-associated nuclear antigen) in BC3 cells, respectively. The capacity of sulfonamides to impair MDM2–p53 complex formation was detected by an enzyme-linked immunosorbent assay method. The analysis of variance was performed according to one-way analysis of variance with Fisher as a *post hoc* test. Here we show that sulfonamide antibiotics are able to suppress the KSHV latent state in permanently infected BC3 lymphoma cells and interfere with the formation of the MDM2–p53 complex that KSHV seemingly needs to support latency and to trigger tumor cell transformation. These findings detected a new molecular target for the activity of sulfonamides and offer a new potential perspective for treating KSHV-induced lymphoproliferative diseases.

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INTRODUCTION

The Kaposi sarcoma herpesvirus (KSHV) is a lymphotropic γ -herpesvirus known as the causative agent of the Kaposi sarcoma (KS) and other lymphoproliferative diseases.^{1,2} Like all the species of herpesvirus, KSHV is also able to cause a lytic infection at first contact, and then to enter into a latent state, whereby the viral episome is bound to cellular DNA by means of the latency factor LANA (latency-associated nuclear antigen).^{3–6}

At present, KS is treated as a malignant tumor with conventional chemotherapeutic agents and/or interferon- α .⁷ Such therapy is often able to control disease evolution, but does not cure the KSHV infection, as the virus can remain in a latent state as an episome bound to cellular DNA for its entire lifespan. In addition, conventional anti-herpes drugs, such as gancyclovir and foscarnet, are able to slightly suppress virus replication in the lytic phase, but do not clear the latent state.^{8,9}

Recent studies have demonstrated that latent herpesviruses, such as Epstein–Barr virus and KSHV, can be inhibited by certain synthetic compounds that are able to interfere with the interaction of the latency factor EBNA1 of Epstein–Barr virus¹⁰ or with the ability of the KSHV latency protein LANA to bind to cellular DNA.¹¹

In this work we found that several sulfonamide antibacterials were able to suppress the KSHV latent state in permanently infected

BC3 lymphoma cells. Studies on the mechanism of action revealed that they could interfere with the formation of the MDM2–p53 complex that KSHV seemingly needs to support latency and to trigger tumor cell transformation.

MATERIALS AND METHODS

Cell culture

KSHV-positive BC3 cells were grown in RPMI-1640 medium with 10% fetal calf serum (Invitrogen, Paisley, UK) as previously described.^{12–14} THP-1 cells (a KSHV-negative macrophage cell line) were purchased from Life Technologies (Paisley, UK). A pool stock of human umbilical vein endothelial cells (primary endothelial human cells from Life Technologies) was grown in M200 medium (Gibco, Paisley, UK) with low serum growth supplement (Invitrogen). The sulfonamide compounds used in this study (see Table 1 and Supplementary File) were commercial drugs purchased from Sigma-Aldrich (St Louis, MO, USA). Nutlin-3a (Sigma-Aldrich) and nimesulide (Sigma-Aldrich) were used as reference drugs. Compound cytotoxicity was evaluated by measuring the effect produced on cell morphology and replication as previously described.¹²

Quantitative PCR (qPCR)

Genomic DNA was isolated using an Easy-DNA kit (Invitrogen) and suspended in 40 μ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA). To detect KSHV DNA, 100 ng of the processed DNA was used as a template, and qPCR was performed on a Real-Time PCR System (StepOne; Applied Biosystems, Monza, Italy) as

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Table 1 Cytotoxic activity of sulfonamide drugs on both human primary and tumor line cells and detection of EC₅₀ and SI on BC3 cells after 6 days of culture

Drugs	CC ₅₀ ^a μg ml ⁻¹ ± s.e.		EC ₅₀ ^b μg ml ⁻¹ ± s.e.			SI (CC ₅₀ /EC ₅₀)
	HUVECs	THP-1	BC3	BC3	BC3	
SG3	500 ± 125	125 ± 31	100 ± 25	21 ± 5	4.76	
SA4	250 ± 125	125 ± 31	100 ± 50	8 ± 4	12.5	
ST5	250 ± 62	30 ± 15	100 ± 25	6 ± 2	16.6	
BC6	125 ± 31	125 ± 62	100 ± 12	12 ± 4	8.3	

Abbreviations: BC3, KSHV permanently infected lymphoblastic cell line; BC6, sulfamethoxazole; HUVEC, human umbilical vein endothelial cell; KSHV, Kaposi sarcoma herpesvirus; SA4, sulfanilamide; SG3, sulfoguanidine; SI, selectivity index; ST5, sulfathiazole; THP-1, KSHV-free macrophagic cell line.

Data are expressed as mean ± s.e. of three independent experiments.

^aCC₅₀: cytotoxic concentration that inhibits cell viability by 50%.

^bEC₅₀: effective concentration was detected by reverse transcription-PCR (RT-PCR) in BC3 cells as inhibition of latent KSHV DNA.

described before.¹⁴ The Custom TaqMan Assay used consisted of KSHV Orf50 primers and probe (forward 5'-CAGCCATATGTAAGCTACT-ACAGCAA-3', reverse 5'-TGTAGAGCTTCAGTAGGGAAGAGGTT-3' and probe 6-FAM-5'-CCTGGAGGCGACTCGTCTGCAATC-3'-MGB).

Cytofluorometry assay

LANA expression was assayed by flow cytofluorometry in BC3 cells with a modification of the method described by Angius *et al.*¹² The cells were incubated at 37 °C and 5% CO₂ for 6 days in the presence of a 100 μM concentration of compounds. Subsequently, cells were washed twice with phosphate-buffered saline and once with RPMI-medium, pH 7.4, and incubated at 37 °C for 1 h with rat anti-LANA antibodies (Santa Cruz Biotech, Dallas, TX, USA) and subsequently with rabbit anti-rat FITC-conjugated antibodies (Sigma-Aldrich). Following incubation, cells were washed with phosphate-buffered saline and suddenly fixed with iced methanol for 10 min at room temperature. After fixation, cells were washed and harvested for measurements. Fluorescence was observed with a Perkin-Elmer (Waltham, MA, USA) flow cytofluorometry apparatus. All data were processed with BD FACSDiva 6.1.3 (Becton Dickinson, Mountain View, CA, USA). At least 10 000 cells were evaluated for each group and each experiment was carried out at least twice. The data are reported as the weighted mean value of fluorescence evaluated in positive cells normalized to the entire population.

MDM2-p53 complex inhibition assay

The interaction of sulfonamide compounds on the MDM2-p53 complex was performed according to the method of Böttger *et al.*¹⁵ on high-binding microtiter-plates (Greiner Bio-One, Kremsmünster, Austria) coated by overnight incubation with 2 μg ml⁻¹ p53 protein (human recombinant, Sigma) in phosphate-buffered saline at 4 °C. The plates were subsequently incubated with MDM2 (1.3 μg ml⁻¹) tagged with glutathione-S-transferase (human GST-MDM2, Sigma, Irvine, UK) prepared in blocking buffer. The experiments were performed in two ways: (1) before incubation: the compounds were added to the p53-coated wells 30 min before adding the GST-MDM2; (2) after incubation: the compounds were added to the wells 30 min after adding GST-MDM2 to the mixture. The plates were then incubated with rabbit anti-GST specific monoclonal antibody (Sigma-Aldrich), followed by incubation with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG rabbit horseradish peroxidase, Sigma). After incubation with stabilized chromogen (Invitrogen), the absorbance was measured with a microtiter plate reader (Model 680, Bio-Rad, Hercules, CA, USA) and the relative luminescence units (RLUs) were measured. The percentage of MDM2-p53 complex inhibition was calculated as the (RLUs detected in the compound treated sample - RLUs of DMSO control)/100. The analysis of variance was performed according to one-way analysis of

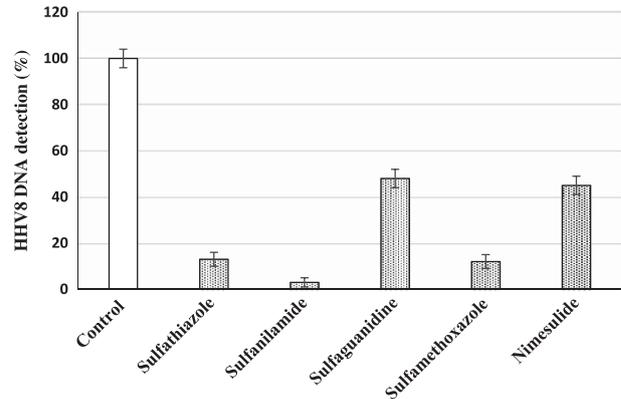


Figure 1 Sulfonamide compounds clear latent Kaposi sarcoma herpesvirus (KSHV) DNA from BC3 cells. Quantitative PCR (qPCR) was applied to the ORF50 KSHV gene for detecting viral DNA in latently infected BC3 cells. The drugs were used at 100 μM concentration (sulfoguanidine (SG3) = 21.4 μg ml⁻¹; sulfanilamide (SA4) = 17.2 μg ml⁻¹; sulfathiazole (ST5) = 25.5 μg ml⁻¹; sulfamethoxazole (BC6) = 25.3 μg ml⁻¹; nimesulide = 30.8 μg ml⁻¹) and left on the cells for 6 days before performing the assay. Nimesulide was used as reference positive control. The data are reported as a percentage of the untreated BC3 control. Data were normalized to 10⁶ cells and are expressed as mean ± s.e. of three independent experiments.

variance with Fisher as a *post hoc* test. A significant difference was considered when *P* < 0.05.

RESULTS

Latent KSHV DNA was detected by qPCR (Figure 1) and the LANA antigen was assayed by a cytofluorometry method (Figure 2). After 6 days of cell culture in the presence of 100 μM drugs (SG3 = 21.4 μg ml⁻¹; SA4 = 17.2 μg ml⁻¹; ST5 = 25.5 μg ml⁻¹; BC6 = 25.3 μg ml⁻¹; see also the Supplementary File), the cell number was counted and a maximum of 15–20% of difference in cell count was recorded in the various plates. We found that the KSHV DNA was cleared from most cells by some of the compounds tested: sulfoguanidine (SG3) inhibited viral DNA by 52 ± 3%, whereas sulfanilamide (SA4), sulfamethoxazole (BC6) and sulfathiazole (ST5) showed antiviral activities of 97 ± 2%, 88 ± 2% and 87 ± 3%, respectively. Nimesulide, known to reduce KSHV latent gene expression, disrupt p53-LANA-1 protein complexes and activate the p53/p21 tumor-suppressor pathway,¹¹ was used as a reference control in our assays, demonstrating an antiviral effect of 55 ± 3% on KSHV DNA.

In this assay we observed an increasing antiviral activity among the tested molecules from sulfoguanidine to sulfathiazole. As a matter of fact, a progressive increase of hydrophobicity from sulfoguanidine to sulfathiazole because of the presence of the heterocyclic moieties was observed, suggesting an increasing affinity to bind to the MDM2-p53 complex, as also indicated by Wang *et al.*¹⁶

Figure 2 shows the inhibition of the LANA expression by BC3 cells treated with sulfonamide compounds at 100 μM concentration. After 6 days of treatment with the sulfonamides, the BC3 cells were separated into two populations: the bottom right sector of the frame indicates BC3+/LANA+, and the upper right shows the cells BC3+/LANA-. In this case too, the drugs significantly inhibited HHV8 latent antigen expression; the final data were ~65 ± 4% of LANA inhibition for SG3 (33% of residual fluorescence (RF)), 69 ± 5% for BC6 (32% of RF), 78 ± 6% for SA4 (21% of RF) and 85 ± 3% for ST5 (17% of RF). In these conditions nimesulide showed a score of 54 ± 5% (~46% of RF). All the data were normalized to 10⁶ cells.

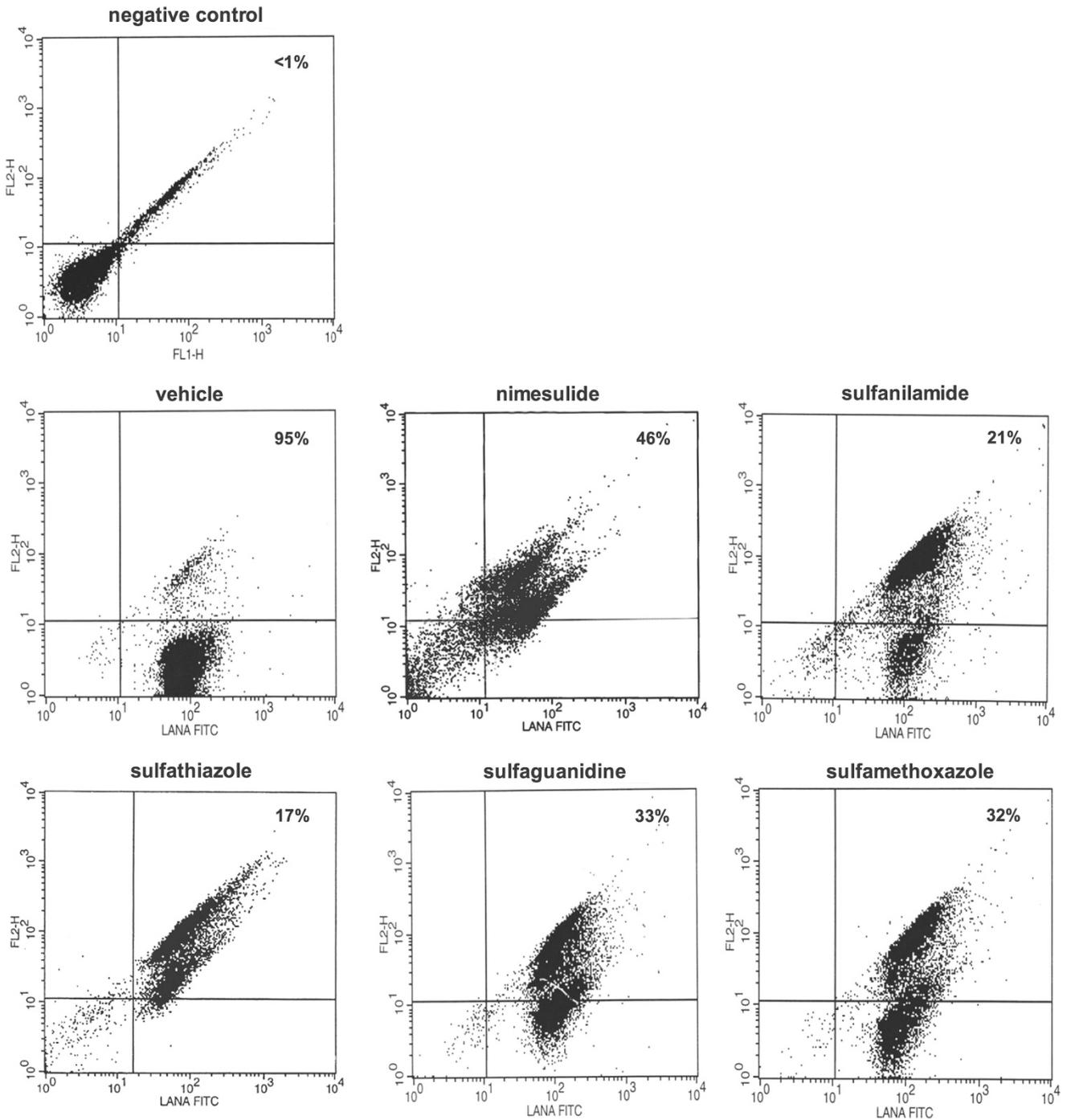


Figure 2 Cytofluorometry assay for detecting the Kaposi sarcoma herpesvirus (KSHV) LANA (latency-associated nuclear antigen) latency antigen in BC3 cells. The numbers in the frame indicate the percentage of residual fluorescence within the drug-treated latently infected BC3 cells and the data were normalized to 10^6 cells. All the drugs were used at the concentration of $100 \mu\text{M}$ and left on the cell culture for 6 days. Each frame was set so that some sectors were identified: the bottom right sector indicates BC3+/LANA+ cells; the upper right shows the cells BC3+/LANA-; the bottom left is a negative control. All the sulfonamide drugs tested scored significant values as compared with the untreated BC3 control ($P < 0.05$).

The cytotoxic activity of the tested drugs was detected on BC3 (KSHV permanently infected lymphoblasts) and THP-1 (KSHV-free macrophages) cell lines, and also on human umbilical vein endothelial cells (primary human umbilical cord cells) after 6 days of culture. Sulfonamide drugs showed a CC_{50} (cytotoxic concentration that inhibits cell viability by 50%) of $\sim 100 \mu\text{g ml}^{-1}$ on BC3 cells (Table 1), whereas the values for CC_{50} on THP-1 varied

from $30 \mu\text{g ml}^{-1}$ (ST5) to $125 \mu\text{g ml}^{-1}$ (SG3, SA4, BC6); primary human umbilical vein endothelial cells appeared generally more resistant to drug cytotoxicity, with CC_{50} of $125 \mu\text{g ml}^{-1}$ (BC6), 250 (SA4 and ST5) and $500 \mu\text{g ml}^{-1}$ (SG3). The drug EC_{50} (half-maximal effective concentration) was detected in BC3 cells and the relative selectivity indexes were 4.76 for SG3, 12.5 for SA4, 16.6 for ST5 and 8.3 for BC6.

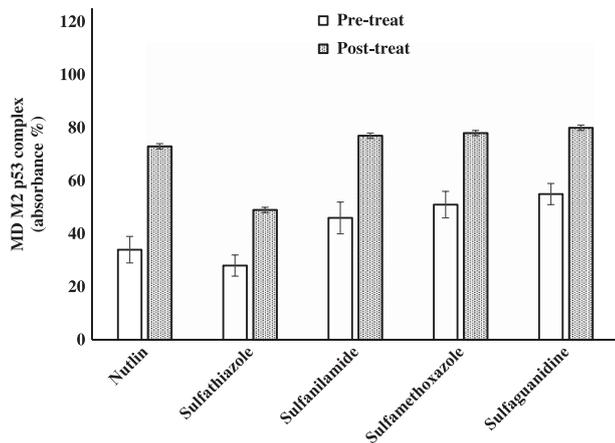


Figure 3 Inhibitory activity of sulfonamide compounds on MDM2–p53 complex formation. Empty columns (Pre-treat): the compounds were added to the reaction mixture before the formation of the MDM2–p53 complex; black columns (Post-treat): the drugs were added to the reaction after the formation of the MDM2–p53 complex. All the compounds were used at a concentration of 100 μM (sulfoguanidine (SG3)=21.4 $\mu\text{g ml}^{-1}$; sulfanilamide (SA4)=17.2 $\mu\text{g ml}^{-1}$; sulfathiazole (ST5)=25.5 $\mu\text{g ml}^{-1}$; sulfamethoxazole (BC6)=25.3 $\mu\text{g ml}^{-1}$; nutlin-3a=58.1 $\mu\text{g ml}^{-1}$). The values are reported as a percentage of the negative control. Nutlin-3a was used as a positive control. Data are expressed as mean \pm s.e. of three independent experiments.

The activity of the sulfonamide drugs was tested on the MDM2–p53 complex before and after its formation. Figure 3 shows that most of the tested compounds significantly inhibited the formation of the MDM2–p53 complex when added to the test wells before its establishment. In comparison with nutlin-3a, used as a positive control, ST5 showed a slightly stronger activity (~70% inhibition compared with 62% of nutlin-3a); SA4 and BC6 also inhibited complex formation by 56% and 50% respectively. Sulfaguanidine was the least active compound in this test, with a score of ~48%. Finally, both nutlin-3a and sulfonamide compounds exerted some activity in disrupting the MDM2–p53 complex after its formation: ST5, the most active drug, disrupted the complex by ~48% ($P < 0.01$), compared with ~28% inhibition exerted by nutlin-3a ($P < 0.05$). All the other sulfonamides showed nonsignificant values in this assay, whereas the difference between pre- and posttreatment was significant ($P < 0.05$) for each compound.

DISCUSSION

Sulfonamide antibiotics were the earliest selective antibacterial drugs to be discovered and used for treating microbial infections. Their capacity to antagonize folic acid synthesis made them one of the most frequently prescribed and widely used antibiotics in the 1930s and 1940s.¹⁷ Afterwards, their use and importance decreased, as more active and less toxic natural antibiotics were discovered. Recently, the worldwide diffusion of bacterial resistance has induced many researchers to reevaluate sulfonamide antibiotics in the treatment of bacterial infections.¹⁸

In this work we show that some of the most common sulfonamide drugs are endowed with strong activity on latently KSHV-infected BC3 cells, thereby allowing them to play a possible potential role in clearing the KSHV-infected lymphoma cells. These compounds interfere with the formation of the MDM2–p53 complex that has been demonstrated to also contain LANA in KSHV-infected BC3 lymphoma cells.¹⁹ MDM2, as well as LANA, also binds to the N terminus of p53.²⁰

This interaction seems to be sensitive to nontoxic doses of sulfonamides. Hence, the nature of the complex must be such as to allow sulfonamides access to the p53 N terminus and the p53–MDM2 interaction site, after which the viral genome detaches from the cellular DNA and is destroyed by DNA polymerases; as a consequence, LANA, which is the specific factor of KSHV latency, also stops being transcribed and is cleared from cell nucleus and cytoplasm. Our findings fit with the model of Chen *et al.*,²⁰ demonstrating that MDM2–p53 disruption via nutlin-3a will also release LANA from p53. Moreover, sulfonamides disrupted purified MDM2–p53 complex *in vitro*, suggesting a direct interaction between these drugs and the MDM2–p53 complex that may have the correct structure to bind LANA.

It is well known that p53 is a key protein for the regulation of cell differentiation.^{19,21} Interaction with the oncogene MDM2 impairs the p53 activity in normal cells, and overexpression of MDM2 can dysregulate cell replication, leading to an oncogenic cell transformation. KSHV sustains the MDM2–p53 complex by adding the LANA protein, thereby abolishing the p53 function, after which the virus remains in a latent state inside the cell as an episome.^{20,22} Both sulfonamides and nutlin-3a are able to reactivate p53, thus enhancing its role as a cell genome regulator.^{16,23} KSHV-associated lymphomas and KS are unusual, as they contain wild-type p53, unlike the majority of nonviral cancers, in which p53 is mutated. This suggests that the MDM2–p53 complex is perhaps activated by KSHV at the protein levels or that some of p53 activities may indeed be beneficial to support latent persistence.^{24,25} The MDM2 gene encodes a nuclear E3 ubiquitinligase that is also transcriptionally regulated by p53. Sulfonamide antibiotics are able to disrupt this complex and reactivate p53, leading to cell differentiation or occasionally to cell apoptosis.²⁶ Moreover, KSHV also targets the p53-related p73 and disruption of this complex can also induce apoptosis.²⁷

Regarding a possible structure–activity relationship, Wang *et al.*¹⁶ demonstrated that the sulfonamide moiety can play an important role in the enhanced potency of the molecules in the MDM2–p53 complex, as the sulfonamide side chain acts as a directing group that orients the side chemical groups into the pocket of the complex. Li *et al.*¹⁰ also analyzed the interaction between EBNA1 (the latent factor of Epstein–Barr γ -herpesvirus) and the MDM2–p53 complex. They found that the sulfonyl motif of the tested sulfonamide derivatives mimics the interaction with the receptor. In particular, compounds with modifications that extend the hydrophobic motifs might enable compound analogs to reach deeper into the EBNA1-binding pocket. Chen *et al.*²⁰ have recently suggested that under normal conditions, nutlin-3a disrupts purified MDM2–p53 complex binding by intercalating at the p53–MDM2 surface. They concluded that MDM2 contributes to LANA interaction with p53 either directly or by changing p53 conformation to enable it to bind LANA. Thus, the MDM2–p53 complex is the key that allows LANA binding to and stabilization on cellular DNA. Disrupting MDM2–p53 automatically induces the termination of HHV8 latency. Therefore, the sulfonamide antibiotics used in this study seem to behave almost similarly to nutlin-3a, by binding to the MDM2–p53 complex that is destabilized and releases latent HHV8 factors; in addition, the different hydrophobicity of the tested sulfonamides can justify the observed increasing inhibitory activity from sulfaguanidine to sulfathiazole.

In conclusion, the overall finding of this work is that some sulfonamide compounds, widely used as antibacterial drugs, can be considered a new potential tool for inactivating KSHV, and thus clearing latent infection specifically in KSHV-infected cells. In addition, sulfonamides inhibit latent KSHV at low μM concentrations

that can easily be reached after standard sulfonamide therapy (sulfamethoxazole serum concentration up to 40–60 $\mu\text{g ml}^{-1}$ after a standard oral dose of 800 mg).²⁸ It would also be interesting to verify whether other herpesviruses could be inhibited in their latent state by these drugs. This finding could open up a very promising program of studies on both oncogenic and degenerative diseases, in which herpesvirus latency is suspected to be involved.²⁹

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contributions: RP, AI and DPD designed and wrote the paper; FA, EP and SU performed reverse transcription-PCR and enzyme-linked immunosorbent assays; CM, RS, RB and WC performed all the fluorescence and cytofluorometry tests.

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