1 **Targeting the Kaposi Sarcoma Herpesvirus ORF 21 tyrosine kinase and viral lytic reactivation**
2 **by tyrosine kinase inhibitors approved for clinical use**

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19 **ABSTRACT**

20 Kaposi's Sarcoma-associated herpesvirus (KSHV) is the cause of three human malignancies,
21 Kaposi's Sarcoma, Primary Effusion Lymphoma and the plasma cell variant of Multicentric
22 Castleman's Disease. Previous research has shown that several cellular tyrosine kinases play
23 crucial roles during several steps in the virus replication cycle. Two KSHV proteins also have
24 protein kinase function: open reading frame (ORF) 36 encodes a serin-threonine kinase, while
25 ORF21 encodes a thymidine kinase (TK), which has recently been found to be an efficient
26 tyrosine kinase. In this study, we explore the role of the ORF21 tyrosine kinase function in KSHV
27 lytic replication. By generating a recombinant KSHV mutant with an enzymatically inactive
28 ORF21 protein we show that the tyrosine kinase function of ORF21/TK is not required for the
29 progression of the lytic replication in tissue culture, but that it is essential for the
30 phosphorylation and activation to toxic moieties of the antiviral drugs zidovudine and
31 brivudine. In addition, we identify several tyrosine kinase inhibitors, already in clinical use
32 against human malignancies, which potently inhibit not only ORF21 TK kinase function, but also
33 viral lytic reactivation and the development of KSHV-infected endothelial tumors in mice. As
34 they target both cellular tyrosine kinases and a viral kinase, some of these compounds might
35 find a use in the treatment of KSHV-associated malignancies.

36 **Importance:** Our findings address the role of KSHV ORF21 as a tyrosine kinase during lytic
37 replication and the activation of prodrugs in KSHV-infected cells. We also show the potential of
38 selected clinically approved tyrosine kinase inhibitors to inhibit KSHV TK, KSHV lytic replication,
39 infectious virions release and the development of an endothelial tumor. Since they target both
40 cellular tyrosine kinases supporting productive viral replication and a viral kinase, these drugs,

41 which are already approved for clinical use, may be suitable for repurposing for the treatment
42 of KSHV-related tumors in AIDS patients or transplant recipients.

43

44

45 INTRODUCTION

46 Kaposi's Sarcoma-associated herpesvirus (KSHV), a γ_2 -herpesvirus, causes Kaposi's sarcoma
47 (KS), primary effusion lymphoma (PEL), and multicentric Castleman disease (MCD). KSHV-
48 related diseases occur frequently in AIDS and other immunocompromised patients and are
49 particularly common in sub-Saharan Africa (1, 2). KSHV can infect endothelial cells, B-cells,
50 monocytes, epithelial cells, and keratinocytes (3). In KS tumors mainly endothelial cells are
51 infected, whereas infected B-cells represent the neoplastic cell type in PEL and MCD. In most
52 infected cells KSHV persists in a latent form that is characterised by the expression of only a few
53 viral genes. Physiological stress signals, such as hypoxia, chemicals like sodium butyrate (SB),
54 tetradecanoyl phorbol acetate (TPA) or ectopic expression of RTA, the product of the viral gene
55 *ORF50*, allow the virus to switch from latency to lytic replication (4–7). During lytic replication,
56 most viral genes are expressed and progeny virions are produced. Low levels of spontaneous
57 viral lytic replication *in vivo* are thought to be important for persistence, dissemination and
58 tumorigenesis (8–14). In addition, a "relaxed" latency program (8, 15) involves the production
59 of several viral lytic proteins with paracrine and angiogenic properties, such as vIL6, vMIP I-III,
60 vGPCR, K1 or K15 (16–32). In some cell types, the expression of a select group of lytic viral
61 proteins such as vIL6 and vIRF3 can occur in the absence of RTA (33–36).

62 Among the lytic viral proteins are two virus-encoded kinases, the viral thymidine kinase (TK,
63 *ORF21*) and the Ser/Thr kinase (vPK, *ORF36*), and KSHV also relies on cellular kinases to regulate
64 its replication cycle (37). Viral TKs are conserved among human α - and γ -herpesviruses and are
65 encoded, respectively, by *UL23* of HSV-1/2, *ORF46* of VZV, *BXLF1* of EBV, and *ORF21* of KSHV
66 (Fig. 1A). They mediate the phosphorylation of pyrimidine nucleosides and prodrugs such as
67 zidovudine (AZT), acyclovir (ACV) and brivudine (BRV) (38, 39), which subsequently undergo
68 additional phosphorylation steps by cellular kinases to generate the active drug capable of
69 inhibiting herpesvirus-specific DNA polymerases and viral DNA synthesis (38). Herpesviral
70 thymidine kinases differ significantly in their ability to phosphorylate nucleosides and their
71 analogues. Several studies have shown that KSHV TK preferentially phosphorylates pyrimidine
72 nucleosides and is unable to efficiently phosphorylate purine analogues that are instead
73 preferentially phosphorylated by PK/ORF36 (39, 40). A combined treatment with high doses of
74 Zidovudine and Valganciclovir, which are phosphorylated and converted to toxic moieties by
75 TK/ORF21 and PK/ORF36 respectively, gave a good clinical response against KSHV multicentric
76 Castelman disease (41, 42). However, KSHV TK/ORF21 has an up to 60-fold higher K_m for
77 thymidine than does HSV-1 TK and therefore phosphorylates thymidine much less efficiently
78 (39). TK may therefore have another role in the viral life cycle and, in fact, has recently been
79 shown to also act as a tyrosine kinase (43). KSHV TK autophosphorylates its tyrosine residues
80 65, 85 and 120 (43, 44), which are located in the extended N-terminal domain that is
81 characteristic for γ -herpesviruses but lacking in α -herpesviruses (Fig. 1A). Phosphorylated TK
82 interacts with and phosphorylates Crk family proteins and the p85 regulatory subunit of PI3-
83 Kinase. When KSHV TK is overexpressed by transfection, autophosphorylation of TK results in

84 the disruption of focal adhesions and activation of Rho-ROCK-Myosin II-dependent cell
85 contraction and blebbing (43). Replacing three essential glycine residues in the ATP binding
86 pocket (GxxGxGK) (Fig. 1A) of the kinase domain with valine leads to a dramatic decrease in TK
87 autophosphorylation (45).

88 HSV-1 TK is important for reactivation from latency (46) and murine γ -herpesvirus 68 (MHV-68)
89 mutants with a disrupted TK gene replicated normally *in vitro* but were severely attenuated *in*
90 *vivo* (47, 48). Here, we show that KSHV TK and its kinase function are dispensable for lytic
91 replication in tissue culture, but required for the activation of the antiviral drugs brivudine and
92 zidovudine in KSHV-infected cells. We also identified several clinically approved tyrosine kinase
93 inhibitors that inhibit TK kinase function and viral lytic reactivation. Several of these inhibitors
94 antagonized the formation of endothelial tumors in a mouse xenograft model with KSHV-
95 infected endothelial cells. These results highlight the potential of selected FDA-approved
96 tyrosine kinase inhibitors to target KSHV TK, inhibit KSHV lytic replication as well as KSHV
97 tumorigenesis.

98

99 RESULTS

100 Mutation of the ORF21/TK ATP-binding pocket in the KSHV genome abrogate TK 101 phosphorylation but does not interfere with the KSHV lytic cycle in tissue culture

102 KSHV TK has been shown to be autophosphorylated on three tyrosine residues located in the
103 extended N-terminal domain of KSHV TK, which is typical for γ -herpesviruses (KSHV, EBV, MHV-
104 68) but lacking in α -herpesviruses (HSV-1,-2, VZV; see Fig. 1A) (43). Replacing three essential

glycine residues in the ATP binding pocket (GxxGxGK) (Fig. 1A) of the kinase domain with valine leads to a dramatic decrease in TK autophosphorylation (43, 49).

To confirm those results, we transfected an expression vector for ORF21/TK, fused to an N-terminal GFP, into HEK293 and analysed cell lysates by immunoblot using an antibody specific for phosphorylated tyrosine residues. This revealed a phosphorylated band of the molecular weight corresponding to that of the GFP-TK fusion protein (Fig. 1B). Immunoprecipitation with an antibody to GFP showed that the phosphorylated band corresponded to the transfected GFP-TK fusion protein (Fig. 1B, top panels). To confirm that this signal is specific for TK phosphorylated on tyrosine residues, we performed a similar experiment using expression vectors for GFP alone, GFP-TK wt and GFP-TK mutants harbouring either 3 point mutations (G₂₆₀V, G₂₆₃V, G₂₆₅V) replacing glycine with valine residues in the ATP binding pocket (GFP-TK 3GV; see Fig. 1A), or 3 point mutations replacing tyrosine residues 65, 85 and 120 in the aminoterminal domain of KSHV TK with phenylalanine residues (GFP-TK 3YF; see Fig. 1A), which have been previously shown to be autophosphorylated in KSHV TK (43, 44). Neither of these mutants was recognized by the antibody to phosphorylated tyrosine residues (Fig. 1C), suggesting that the observed phosphorylation of wt TK (Fig. 1B) is mainly due to autophosphorylation and that cellular kinases are not involved in this process.

We next addressed the role of the ORF21 tyrosine kinase function during viral lytic replication. Using the KSHV BAC16 genome, we created a KSHV mutant harbouring the above three point mutations (G₂₆₀V, G₂₆₃V, G₂₆₅V) in the ATP binding pocket, which inhibit the TK tyrosine kinase function (43) (Fig. 1B and 1C). iSLK cells were transfected with wt KSHV BAC and this KSHV BAC mutant (3GV) and stably transfected bulk populations were selected. Lytic replication was then

127 induced in these cells by treatment with doxycycline and Na-butyrate, cells were collected 48h
128 after reactivation, lysed and immunoprecipitated with an antibody recognising phosphotyrosine
129 (pY) residues. By immunoblotting with the same anti-phosphotyrosine antibody, we observed
130 an increased phosphorylation of a protein band of the molecular weight expected for TK (pTK,
131 approximately 65 kd) (43, 44) in induced iSLK/BAC16 wt, but not in induced iSLK/BAC16 3GV
132 cells (Fig. 1D). Similar results were obtained with three independently generated sets of iSLK
133 populations that had been stably transfected with KSHV-wt and KSHV-ORF21_3GV bacmids.
134 This pattern suggests that the phosphorylation of this protein during lytic reactivation is
135 dependent on the TK tyrosine kinase function. We observed that expression of the early lytic
136 proteins ORF45 and K-bZIP were moderately decreased (Fig. 1E), but the titre of viral progeny
137 not affected (Fig. 1F), if the TK tyrosine kinase function was abolished. We conclude that the
138 enzymatic function of KSHV ORF21/TK is required for efficient TK autophosphorylation in KSHV-
139 infected cells undergoing lytic replication, but is not essential for the KSHV lytic cycle in cultured
140 epithelial cells.

141

142 **ORF21/TK is required to activate Brivudine and Zidovudine in KSHV-infected cells**

143 Pyrimidine nucleoside derivatives have been shown to be preferentially phosphorylated by
144 herpesviral thymidine kinases and purine nucleosides are preferentially activated by beta- and
145 gammaherpesvirus protein kinases (40). Based on this evidence we tested two pyrimidine
146 compounds, brivudine and zidovudine, at several concentrations in iSLK cells stably infected
147 with either KSHV-wt or the KSHV-ORF21_3GV mutant. Following induction of the lytic cycle, the
148 production of infectious progeny virus was inhibited by both drugs in a dose-dependent

149 manner in KSHV-wt-, but not in KSHV-ORF21_3GV-infected cells, suggesting that the KSHV-
150 ORF21_3GV mutant is resistant to both compounds (Fig. 2A and 2B). Cell viability, evaluated
151 using an MTT assay, strongly decreased with increasing concentrations of brivudine and
152 zidovudine for iSLK/KSHV-wt, but only moderately for iSLK/KSHV-ORF21_3GV cells (Fig. 2C and
153 2D), suggesting that KSHV TK promotes the conversion of brivudine and zidovudine into cytotoxic
154 moieties in KSHV-infected cells.

155

156 **Tyrosine kinase inhibitors approved for clinical use inhibit KSHV TK (ORF21)** 157 **autophosphorylation**

158 We next explored if tyrosine kinase inhibitors that are approved by the Food and Drug
159 Administration (FDA) for clinical use could inhibit the ORF21/TK tyrosine kinase activity.

160 Using the assay performed in Fig. 1B, we investigated whether any of 27 FDA-approved kinase
161 inhibitors (Table 1) are able to inhibit KSHV ORF21/TK autophosphorylation. Twenty-four hours
162 after transfection, HEK293 cells expressing GFP-TK were treated with 1 μ M of each inhibitor
163 (Fig. 3). We observed that the level of autophosphorylated TK was markedly (at least 80%)
164 decreased by bosutinib, dasatinib and ponatinib, and to a lesser extent by gefitinib and nilotinib
165 (Fig. 3). These results suggest that these drugs are able to inhibit ORF21/TK kinase activity.

166 We confirmed these results in an *ex cellulo* assay, in which we performed a kinase assay on
167 immunoprecipitated GFP-TK. Forty-eight hours after transfecting HEK293 cells with GFP-TK as
168 above, GFP-TK was immunoprecipitated with an antibody to GFP and immune complexes were
169 incubated, in the presence or absence of ATP, with bosutinib, dasatinib, ponatinib, gefitinib and
170 nilotinib, or DMSO as solvent control. Autophosphorylation levels of TK were estimated by

171 immunoblot using an anti-phospho-tyrosine antibody. The results indicate that the tested
172 compounds inhibit autophosphorylation of immunoprecipitated TK *in vitro* in the absence of
173 other cellular proteins (Fig. 4A). To exclude the possibility that an associated cellular kinase
174 might have co-immunoprecipitated with ORF21/TK and caused its phosphorylation, we
175 analysed the ability of the kinase dead mutant GFP-TK 3GV to autophosphorylate under the
176 same conditions (Fig. 4A). We observed no significant phosphorylation with this mutant,
177 indicating that the observed phosphorylation is mainly due to autophosphorylation of TK. A
178 similar experiment was performed using increasing concentrations of inhibitors (Fig. 4B). We
179 observed that, in contrast to imatinib, which did not show significant inhibition, dasatinib
180 strongly inhibits the autophosphorylation of TK, even at 0.3 μ M. Similar results were observed
181 for bosutinib and ponatinib. In this kinase assay performed with immunoprecipitated TK,
182 ibrutinib, which did not inhibit TK autophosphorylation in transfected cells at 1 μ M (Fig. 3), was
183 able to inhibit TK autophosphorylation by more than 50% of at concentration of 2.5 μ M and
184 higher (Fig. 4B).

185

186 **Kinase inhibitors inhibit TK autophosphorylation in KSHV-infected cells**

187 We further analysed the impact of selected kinase inhibitors on TK autophosphorylation in
188 KSHV-infected cells. Following induction of lytic replication in iSLK/KSHVwt and treatment for
189 48h with 5 or 10 μ M of dasatinib (Fig. 5A), bosutinib (Fig. 5B) or ponatinib (Fig. 5C), we
190 immunoprecipitated phosphorylated proteins as in Fig. 1D. The phosphorylated band
191 corresponding to TK was abolished by all three inhibitors at both concentrations used (Fig. 5A
192 to 5C). All three compounds also strongly inhibited the production of infectious virus (Fig. 5D).

193 Since a KSHV mutant with an enzymatically inactive TK was not strongly compromised in
194 reactivation from latency and production of infectious viral progeny (Fig. 1D to 1F), it is unlikely
195 that the ability of these three tyrosine kinase inhibitors to inhibit virus production was linked to
196 their inhibition of the TK autophosphorylation (Fig. 3 to 5). To confirm this conclusion, we
197 compared the concentrations of dasatinib required to inhibit KSHVwt and the KSHV-3GV
198 mutant carrying an enzymatically inactive TK gene (see Fig. 1D). As shown in Fig. 5E, the KSHV-
199 3GV mutant was still inhibited by dasatinib in a dose-dependent manner, in contrast to its
200 resistance to brivudine (Fig. 2). This suggests that the ability of dasatinib and other tyrosine
201 kinase inhibitors to inhibit KSHV reactivation is likely due to their inhibition of cellular tyrosine
202 kinases.

203

204 **Inhibition of KSHV lytic reactivation by FDA-approved kinase inhibitors in different cell types**

205 To further explore the inhibition of KSHV reactivation by FDA-approved kinase inhibitors, we
206 tested their effect on KSHV lytic replication in B cells (BJAB-rKSHV.219), endothelial cells
207 (HuARLT-rKSHV.219) and another epithelial cell (Vero-rKSHV.219), in which the lytic cycle was
208 activated by different means. In BJAB-rKSHV.219 cells, the lytic cycle was induced using an
209 antibody to IgM (50), and in HuARLT-rKSHV.219 and Vero-rKSHV.219 with a baculovirus
210 expressing RTA together in combination with Na-butyrate. The titre of infectious KSHV released
211 from BJAB-rKSHV.219 was measured by infecting HEK293 cells as described above and the
212 strength of the GFP signal in infected HEK293 cells was quantified by fluorimeter 48h after
213 infection (Fig. 6A). For the HuARLT-rKSHV.219 (Fig. 6B) and Vero-rKSHV.219 (Fig. 6C), the
214 number of RFP expressing cells, *i.e.* cells undergoing lytic reactivation, was determined by

215 microscopy 48h after treatment. Inhibitor-treated cells were stained with MTT (BJAB-
216 rKSHV.219) or DAPI (HuARLT-rKSHV.219, Vero-rKSHV.219) to assess cell viability.

217 Dasatinib, ponatinib, ibrutinib, cabozantinib, lenvatinib and trametinib inhibited virus release
218 from induced BJAB-rKSHV.219 cells at non-toxic concentrations (Fig. 6A). Crizotinib, pazopanib
219 and regorafenib inhibited to a lesser extent in this assay. Dasatinib, ponatinib, ibrutinib and
220 trametinib also lowered the expression of the KSHV early protein K-bZIP in BJAB-rKSHV.219 cells
221 (Fig. 7A). Bosutinib, an inhibitor of ORF21/TK kinase activity (Fig. 3 and 4), inhibited virus
222 release in KSHV-infected endothelial (HuARLT-rKSHV.219) and epithelial (Vero-rKSHV.219) cells
223 (Fig. 6B and 6C), but increased K-bZIP expression (Fig. 7A) and virus production (Fig. 6A) in BJAB-
224 rKSHV.219 cells. Trametinib, a serine/threonine kinase inhibitor, inhibits the lytic replication
225 cycle without inhibiting TK autophosphorylation (Fig. 3, 6A and 7A).

226 The fact that dasatinib, ponatinib, ibrutinib, trametinib decreased the expression of the early
227 KSHV protein K-bZIP in KSHV-infected cells (Fig. 7A) indicates that they act at the level of virus
228 reactivation. To assess if they might also inhibit viral entry, we tested several FDA-approved
229 kinases inhibitors in a KSHV entry assay. We infected HEK293 cells with a stock of KSHV.219,
230 applied the inhibitors at two concentrations (1 and 10 μ M) at the time of infection and scored
231 the number of GFP-positive HEK293 cells after 48 hours. We observed a moderate inhibition at
232 10 μ M for bosutinib, ponatinib and dasatinib, while only ponatinib inhibited KSHV entry at 1
233 μ M (Fig. 7B). Since dasatinib potently inhibits K-bZIP expression already at 1 μ M (Fig. 7A), and
234 inhibits virus production at concentrations well below 1 μ M (Fig. 5E), we conclude that it
235 inhibits KSHV mainly at the stage of lytic cycle induction.

236

237 Inhibition of KSHV-induced tumor formation by FDA-approved kinase inhibitors

238 To assess an effect of selected inhibitors on KSHV-induced tumor formation *in vivo*, we
239 implanted HuARLT-rKSHV spheroids (51) subcutaneously into mice and treated these with
240 dasatinib, ibrutinib, imatinib or ponatinib via oral gavage. Four weeks later, the diameter of
241 tumors was measured. We observed that dasatinib and imatinib were as potent as rapamycin
242 in this mouse model and reduced the tumour diameter by around 25% (Fig. 8).

243

244 DISCUSSION

245 In this study, we explored the role of the recently described (43) tyrosine kinase function of the
246 KSHV ORF21/thymidine kinase in KSHV lytic replication and show that it can be inhibited by
247 several tyrosine kinase inhibitors that are already approved for clinical use. Replacing WT
248 ORF21 with a "kinase-dead" triple mutant ORF21 gene in a recombinant KSHV genome (Fig. 1D
249 to 1F) did not strongly interfere with the lytic replication in cultured epithelial cells (iSLK cells).
250 This result is in line with the previously reported observation that MHV68 ORF21/TK is
251 dispensable for productive MHV68 replication in cell culture, but required for viral productive
252 replication in the lungs of infected mice (47, 48). Therefore, it is conceivable that the tyrosine
253 kinase function of KSHV ORF21/TK may play a role for KSHV replication in infected patients. In
254 cells transfected with an ORF21/TK expression vector, its tyrosine kinase function contributes to
255 morphological changes in ORF21/TK-expressing cell (43). In our experiments with the KSHV-
256 ORF21_3GV virus, whose tyrosine kinase function is deficient, we did not observe any changes
257 in cell morphology between KSHVwt- and KSHV-ORF21_3GV-infected cells. This may be due to
258 the lower level of expression of ORF21/TK in KSHV-infected as opposed to ORF21/TK-

259 transfected cells or the fact that there were relatively few cells undergoing KSHV lytic
260 reactivation in our induced cultures.

261 TK preferentially phosphorylates pyrimidine nucleosides and it is unable to efficiently
262 phosphorylate purine analogues (39, 40). When MHV68 and Herpesvirus saimiri (HVS) were
263 passaged repeatedly in the presence of brivudine, resistance mapped to mutations in their
264 ORF21/TK genes (40). In keeping with this observation, we show here that a 'kinase-dead' KSHV
265 mutant, KSHV-ORF21_3GV, is resistant to brivudine (Fig. 2A and 2C), underlining the
266 importance of KSHV ORF21/TK for the activation of brivudine in infected cells to become an
267 effective antiviral drug. We found the same for zidovudine (Fig. 2B and 2D). This observation is
268 in line with the successful use of high dose zidovudine in combination with valganciclovir to
269 treat patients with active multicentric Castelman disease (41, 42).

270 In view of the tyrosine kinase function of KSHV ORF21/TK and the essential role of MHV-68 TK
271 for virus replication *in vivo* (47, 48), we next explored if tyrosine kinase inhibitors that are
272 already available for clinical use could inhibit the enzymatic activity of KSHV ORF21/TK. We
273 screened FDA-approved inhibitors of cellular tyrosine kinases for compounds that are able to
274 inhibit the enzymatic activity of ORF21/TK and/or KSHV lytic replication. We identified
275 bosutinib, dasatinib and ponatinib as potent inhibitors of ORF21/TK autophosphorylation in
276 ORF21/TK-transfected cells (Fig. 3), in an *in vitro* kinase assay using immunoprecipitated
277 ORF21/TK (Fig. 4) and in KSHV-infected iSLK/BAC16 cells (Fig. 5). In ORF21/TK-transfected cells,
278 or against immunoprecipitated ORF21/TK, gefitinib, nilotinib and nintedanib showed a
279 moderate inhibition of ORF21/TK autophosphorylation (Fig. 3 and 4). While the two most
280 potent inhibitors of ORF21/TK autophosphorylation, dasatinib and ponatinib, all strongly

281 inhibited KSHV lytic replication in B-, endothelial and epithelial cells and expression of the KSHV
282 early protein K-bZIP, the three less potent compounds gefitinib, nilotinib and nintedanib did not
283 (Fig. 6 and 7). Dasatinib was not toxic in any of the three cell lines tested, while bosutinib and
284 ponatinib showed some toxicity in KSHV-infected BJAB and Vero cells (Fig. 6).

285 Four other cellular tyrosine kinase inhibitors, ibrutinib, lenvatinib, cabozantinib and pazopanib
286 also inhibited KSHV lytic replication at non-toxic concentrations in at least one of the three
287 tested cell lines without inhibiting ORF21/TK enzymatic activity (Fig. 3, 4, 6 and 7). As expected,
288 trametinib, a serine/threonine inhibitor (Table 1), did not inhibit ORF21/TK
289 autophosphorylation (Fig. 3 and 4), but potently inhibited KSHV lytic reactivation (Fig. 6 and 7).

290 The observation that the 'kinase-dead' KSHV-ORF21_3GV mutant was not compromised in its
291 ability to undergo lytic reactivation (Fig. 1D to 1F) suggested that the antiviral properties in
292 tissue culture of dasatinib, ponatinib and bosutinib (Fig. 5 to 7) are not dependent on their
293 ability to inhibit ORF21/TK autophosphorylation (Fig. 3 and 4). We confirmed this conclusion by
294 showing that the KSHV-ORF21_3GV mutant is still susceptible to inhibition by dasatinib (Fig.
295 5E). The ability of these tyrosine kinase inhibitors to target cellular kinases is therefore the most
296 likely explanation for their antiviral properties in tissue culture. However, taken together, our
297 results suggest that, in patients with active KSHV replication, as is observed in cases of florid KS
298 (52, 53) and MCD (54–56), dasatinib or other tyrosine kinase inhibitors with activity against
299 ORF21/TK might have a dual effect, targeting both cellular tyrosine kinases and the kinase
300 function of ORF21/TK, which is, at least in the case of the animal model provided by MHV-68,
301 required for *in vivo* lytic replication (47, 48).

302 There is already clinical evidence that tyrosine kinase inhibitors may play a role in the treatment
303 of KSHV-associated disease. Imatinib mesylate has been used in phase I and II clinical trials in
304 AIDS-related cutaneous KS (NCT00090987) and achieved a partial regression of cutaneous KS
305 lesions in some patients (57, 58). Inhibition of c-kit and/or PDGF receptors by imatinib may
306 have contributed to this observation (59, 60). However, imatinib does not inhibit the tyrosine
307 kinase function of KSHV ORF21/TK (Fig. 3 and 4) and was less efficient in inhibiting the KSHV
308 lytic replication than dasatinib, bosutinib, ponatinib and ibrutinib (Fig. 6 and 7). It is therefore
309 unlikely to target KSHV directly *in vivo*.

310 Dasatinib is more potent than imatinib in inhibiting c-Kit (K_d of 0.85 nM and 13 nM,
311 respectively) and PDGFR β (K_d of 0.63 nM and 14 nM respectively) (61, 62) and also targets Abl,
312 EphA2 and Src family kinases (SFKs). By targeting SFKs and PDGFR β , dasatinib inhibits several
313 pathways which are activated during the KSHV life cycle (63–65). Dasatinib also inhibits EphA2,
314 a receptor tyrosine kinase (RTK) involved in KSHV entry and signaling (66–69). Our results
315 suggest that it is also a potent inhibitor of ORF21/TK phosphorylation and KSHV lytic
316 reactivation in three different cell types without apparent cell toxicity. Dasatinib could
317 therefore be a promising candidate to be evaluated as a treatment of KS or MCD. Dasatinib has
318 already been used in a Phase I clinical trial to treat patients with lymphoma, including EBV-
319 associated lymphoma (NCT00608361).

320 Like dasatinib, ponatinib is a multi-target kinase inhibitor. Even though ponatinib strongly
321 inhibits KSHV replication, it was toxic in our assays and its use in patients with CML is known to
322 be associated with occasional severe side effects (70).

323 Bosutinib, the third potent inhibitor of ORF21/TK, inhibited lytic replication in KSHV-infected
324 endothelial and epithelial cells, but increased virus production in anti-IgM stimulated KSHV-
325 infected BJAB cells, raising the possibility that it might promote the reactivation of KSHV from
326 latently infected B-cells *in vivo*. We hypothesize that bosutinib may also target cellular kinases
327 that downregulate lytic replication in B cells. As reported previously, cellular Tausled-like
328 kinases (TLKs) repressed KSHV reactivation from latency (71).

329 Ibrutinib mainly targets the Bruton's tyrosine kinase (BTK), a non-receptor tyrosine kinase
330 involved in the BCR signaling pathway, which is known to activate KSHV from latency in B-cells
331 (50). In our experimental systems, ibrutinib potently inhibited KSHV reactivation not only from
332 B-cells, but also endothelial and epithelial cells (Fig. 6 and 7). It showed moderate activity
333 against the ORF21/TK tyrosine kinase function (Fig. 4). Ibrutinib has already been used in a
334 phase III clinical trial (NCT01109069) in patients with chronic lymphocytic leukemia and B cell
335 lymphoma, including EBV-associated lymphoma (72) and may therefore be another candidate
336 drug against KS that would merit evaluation in clinical trials.

337 Sorafenib, a dual tyrosine and serine/threonine protein kinase inhibitor, mainly targets VEGFR,
338 PDGFR and Raf kinases and has been used in a phase I/II clinical trial to treat KSHV associated
339 cancer (NCT00287495). It also inhibits the HCMV replication cycle (73). Sorafenib showed a
340 moderate inhibition of KSHV lytic replication in BJAB-rKSHV.219 cells (μ M range) but was toxic
341 at higher concentrations (Fig. 6).

342 We also evaluated the ability of dasatinib, ibrutinib and ponatinib to inhibit the growth of
343 tumors developing after implantation of HuARLT-rKSHV spheroids into mice. We used
344 rapamycin and imatinib as a reference, as these compounds have been shown to ameliorate

345 post-transplant and AIDS-KS in clinical trials (57, 58, 74, 75). We observed that dasatinib
346 reduced tumor size in implanted mice to approximately the same degree as imatinib and
347 rapamycin (Fig. 8).

348 Unlike ganciclovir, which targets the viral DNA polymerase and thereby a late stage of viral
349 replication and which has previously been shown to reduce the risk for the subsequent
350 emergence of KS in AIDS patients treated for CMV (76), the tyrosine kinase inhibitors identified
351 in this study inhibit the early phase of lytic replication. This would have the advantage of
352 decreasing the expression of early KSHV proteins with paracrine and angiogenic properties that
353 are thought to contribute to KSHV pathogenesis, such as vIL6, vMIP I-III, vGPCR, K1, K15 (16, 17,
354 25, 77–83).

355 In conclusion, we show that several tyrosine kinase inhibitors, including dasatinib, ibrutinib,
356 ponatinib are inhibitors of both ORF21/TK tyrosine kinase activity and KSHV lytic replication.
357 While their antiviral effect in tissue culture is mostly due to their ability to inhibit cellular
358 tyrosine kinases, these compounds could have the advantage of targeting cellular tyrosine
359 kinases as well as a viral kinase that, at least in the animal model provided by MHV-68, has
360 been shown to be required for productive viral replication *in vivo*. Of these three drugs,
361 dasatinib also showed efficacy against a KSHV-induced tumor in a mouse model and may
362 therefore represent a candidate for drug repurposing to be evaluated in clinical trials of KSHV-
363 associated disease.

364

365 MATERIALS AND METHODS

366 Cell culture, transfection, virus production

HEK293, Vero (84), Vero-rKSHV.219 (85), BJAB (86), BJAB-rKSHV.219 (50, 85), HuARLT (87) and HuARLT-rKSHV.219 cells (synonym: HuAR2T and HuAR2T-KSHV) (88, 89), SLK (90, 91), iSLK cells were cultured as described. The production of the recombinant baculovirus expressing KSHV ORF50/RTA used to trigger lytic reactivation was described previously (85). HEK293 cells were transfected in six well plates with 1 µg of each construct using the FuGENE transfection reagent (Promega) at a ratio 3 µl:1 µg of DNA and harvested 48h after transfection.

A rKSHV.219 virus stock was produced from BJAB infected with rKSHV.219 as previously described (92). iSLK cells stably transfected with KSHV BAC16 WT or 3GV were induced with SB (2 mM) and Doxycycline (2 µg/mL). To determine the titer of the virus produced by iSLK/BACs or BJAB-rKSHV.219, the supernatant collected 48h after reactivation/treatment was used to infect HEK293 cells in a 96 well plate (3×10^4 cells/well). The plate was centrifuged for 30 min at 32 °C and 450 g. 72 h later, infected GFP positive HEK293 cells were counted and the viral titer was calculated.

380

381 **Mutagenesis of KSHV BAC16 and generation of KSHV BAC16 stable cell lines**

The following primers: 5'-ACC GTG GAC TAC AGG AAT GTT TAT TTG CTT TAC TTA GAG GTG GTA ATG GTT GTG GTC AAA TCA ACG CTG GTC AAC GGA CGC ATC GTG GCC GGA TCT C-3' and 5'-GGG CAA GAT CCC GCA CAC GGC GTT GAC CAG CGT TGA TTT GAC CAC AAC CAT TAC CAC CTC TAA GTA AAG CAA ATA AGT GAC CAC GTC GTG GAA TGC-3' were used to insert three point mutations ($G_{260}V$, $G_{263}V$, $G_{265}V$) into the ATP binding pocket of the ORF21 gene in the KSHV genome following the "En passant" protocol (93). This mutant was named KSHV BAC16 3GV. Two individual clones from the first recombination step were selected and used for the second

389 recombination step. New KSHV BAC16 constructs were confirmed by enzymatic digestion and
390 completely sequenced on an Illumina MiSeq sequencer. The sequence of the KSHV BAC16 3GV
391 mutant was deposited in GenBank (accession number MN752405). To generate stable
392 iSLK/BAC16 cells, iSLK cells were transfected with the KSHV BAC16 WT or BAC16 3GV DNA using
393 Eugene 6 (Roche #11814443001) and selected with 1.2 mg/mL hygromycin B for 2-3 weeks and
394 maintained as a polyclonal culture with 100 µg/mL hygromycin B (94).

395

396 **Expression plasmid**

397 GFP-TK was obtained by cloning KSHV ORF21 in the pEGFP-C2 vector (Clontech). ORF21 was
398 amplified by PCR from KSHV WT BAC16 using the following primers: 5'-CAC CTC GAG AGC AGA
399 AGG CGG TTT TG-3' which contains a XhoI restriction site and 5'-GCC GAA TTC CTA GAC CCT
400 GCA TGT CTC CTC-3' containing an EcoRI site. The amplified product and pEGFP-C2 were
401 digested by EcoRI and XhoI prior to ligation.

402 A GFP-TK 3GV mutant containing three point mutations (G₂₆₀V, G₂₆₃V, G₂₆₅V) in the ATP binding
403 site was generated by PCR-based mutagenesis using the following primers: 5'- GCT TTA CTT
404 AGA GGT GGT AAT GGT TGT GGT CAA ATC AAC GCT GGT CAA C-3' and 5'- GTT GAC CAG CGT
405 TGA TTT GAC CAC AAC CAT TAC CAC CTC TAA GTA AAG C -3'. A triple mutant of three tyrosine
406 residues in GFP-TK (Y₆₅F, Y₈₅F, Y₁₂₀F) was also generated by PCR-based mutagenesis using the
407 primers for Y₆₅F (5'- CCT CGT ACA TAT TCG ACG TGC CCA CCG -3'; 5'- CGG TGG GCA CGT CGA
408 ATA TGT ACG AGG-3'), Y₈₅F (5'- TGC ACG ACA ACT CCC TCT TTG CAA CGC CTA GGT TTC CGC C-3';
409 5'- GGC GGA AAC CTA GGC GTT GCA AAG AGG GAG TTG TCG TGC A-3') and Y₁₂₀F (5'- TGA CGA
410 CGA CTC GGG AGA CTT TGC GCC AAT GGA TCG CTT C -3'; 5'- GAA GCG ATC CAT TGG CGC AAA

411 GTC TCC GAG TCG TCG TCA -3'). The presence of the correct mutation and absence of
412 unintended mutations was checked by Sanger sequencing.

413

414 **Immunoblotting, Antibodies, Immunoprecipitation, Kinase Assay**

415 For immunoblotting, cells were lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v)
416 SDS, 10% glycerol, 50 mM DTT, 0.01% (w/v) bromophenol blue) and immunoblots performed
417 using standard protocols. Proteins were detected by the following primary antibodies: mouse
418 monoclonal HHV-8 KbZIP antibody F33P1 (sc-69797, Santa Cruz), mouse monoclonal KSHV
419 ORF45 (sc-53883, Santa Cruz), rat monoclonal KSHV ORF73 (LNA-1) antibody (13-210-100,
420 Advanced Biotechnologies), rabbit monoclonal GAPDH antibody (#2118, Cell Signaling
421 Technology), mouse monoclonal β -actin (#A5441, Sigma), mouse monoclonal Phospho-Tyrosine
422 (P-Tyr-100) (Cell Signaling Technology), mouse monoclonal GFP antibody (#632380, Clontech),
423 rabbit polyclonal GFP antibody (#632592, Clontech).

424 For immunoprecipitation of GFP-tagged ORF21/TK, HEK293 cells were collected forty-eight
425 hours after transfection and lysed for 1 hour on ice with lysis buffer (10 mM Tris-HCl pH 7.4,
426 150 mM NaCl, 5 mM EDTA, 10% Glycerol, 1% Triton X-100) supplemented with cOmplete Ultra
427 protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche).
428 Lysates were centrifuged at 20,000g at 4°C for 10 min and the resulting supernatants were
429 gently shaken overnight at 4°C with Protein G Sepharose 4 Fast Flow beads (GE Healthcare) and
430 rabbit polyclonal GFP antibody (#632592, Clontech) diluted to 1/500. Beads were washed three
431 times with lysis buffer and analyzed by SDS-PAGE and immunoblotting. A similar protocol was

432 used for immunoprecipitation of induced/treated iSLK/KSHV BAC16 WT and iSLK/KSHV BAC16
433 3GV.

434 For the kinase assay, beads obtained after immunoprecipitation of GFP-TK were washed twice
435 with lysis buffer followed by a single wash with 25 mM Hepes pH 7, 50 mM NaCl, 2.5 mM EDTA
436 and 2.5 mM DTT. Beads were incubated for 30 min at 30°C and washed with a kinase assay
437 buffer (10 mM Hepes pH 7.7, 75 mM NaCl, 5 mM EDTA and 0.5 mM DTT) supplemented with
438 PhosSTOP phosphatase inhibitor cocktail (Roche). Beads were then incubated for 30 min at
439 30°C in the presence of 0.4 μ M ATP and the FDA-approved kinase inhibitors diluted in kinase
440 assay buffer and the reaction stopped with 5x SDS sample buffer (250 mM Tris-HCl, pH 6.8,
441 10% (w/v) SDS, 50% glycerol, 250 mM DTT, 0.05% (w/v) bromophenol blue).

442

443 **Inhibition of KSHV lytic replication by kinase inhibitors and cell viability assay**

444 For BJAB-rKSHV.219, 1.5×10^4 cells per well were plated in 384-well plates. Twenty-four hours
445 later, cells were treated with kinase inhibitors and triggered for lytic reactivation for 72h as
446 previously described (92, 95). Supernatants from BJAB-rKSHV.219 cells were collected and used
447 to infect fresh HEK293 cells that had been seeded into a 384 well plate 24h before at a density
448 of 4×10^3 cells per well. HEK293 cells were then centrifuged 30 min at 30°C and 450 g and
449 incubated at 37°C for 72h. Cells were fixed with glutaraldehyde and wash with PBS. *De novo*
450 infection was evaluated by the mean fluorescence intensity of GFP-positive HEK293 cells
451 quantified in a BioTek luminometer. HuARLT-rKSHV.219 (1.3×10^4 cells/well) or Vero-rKSHV.219
452 (5×10^3 cells/well) were plated in 96-well plates, and 24 hours later, treated with kinase
453 inhibitors and a baculovirus expressing the KSHV RTA protein and 1.25 mM Na-butyrate for 48h

454 to activate lytic replication. Following fixation with glutaraldehyde, KSHV lytic reactivation was
455 evaluated by quantifying the number of RFP-positive cells using CellProfiler (96).
456 Cell viability after inhibitor treatment was estimated using an MTT assay for BJAB-rKSHV.219
457 cells, or DAPI staining after glutaraldehyde fixation for HuARLT-rKSHV.219 and Vero-rKSHV.219 .
458 Afatinib, axitinib, bosutinib, cabozantinib, ibrutinib, lenvatinib, ponatinib, regorafenib,
459 ruxolitinib phosphate, sorafenib, tofacitinib, vandetanib and vemurafenib were obtained from
460 AbMole. Dabrafenib, nintedanib, motesanib diphosphate, palbociclib and trametinib were
461 obtained from Selleckchem. Anhydrous dasatinib was purchased from Santa Cruz
462 Biotechnology. Crizotinib, imatinib mesylate and sunitinib malate were from Sigma. Ceritinib,
463 erlotinib, gefitinib, lapatinib ditosylate, nilotinib, pazopanib were from Chemietek. All
464 compounds were dissolved at 20 mM in DMSO, except for erlotinib, imatinib mesylate,
465 palbociclib, which were dissolved in water. Erlotinib, lapatinib ditosylate which were diluted to
466 1 mM and 5 mM, respectively. After reconstitution, the inhibitors were stored at -20°C
467 protected from light. Cells were treated with compounds at concentrations indicated in the
468 figure legends and controls contained equivalent amounts of DMSO (Applichem). DMSO
469 concentrations did not exceed 0.1%.
470 iSLK cells stably transfected with KSHV BAC16 WT or 3GV were induced with SB (2 mM) and
471 Doxycycline (2 µg/mL) and treated with 0,01 µM up to 10 µM of Dasatinib (Abmole) or
472 Brivudine (Abmole) or Zidovudine (Sigma) . DMSO treated non-reactivated and reactivated cells
473 were used as control. To determine the production of infectious viral particles, the cell culture
474 supernatants of the iSLK cell lines were centrifuged for 5 min at 2,500 rpm at 4 °C and added to
475 3×10^4 HEK293 cells in a 96 well plate, which had been seeded the day before. The plate was

476 centrifuged for 30 min at 32°C and 450 g. 48 h post infection, cells were fixed with PFA and viral
477 titers were calculated by counting GFP positive cells per well using a Citation 5 Cell Imaging
478 Multi-Mode Reader from BioTek. Cell viability after inhibitor treatment was estimated using an
479 MTT assay 48h post treatment.

480

481 **Entry assay on HEK293**

482 HEK293 cells were infected with rKSHV.219 (MOI 1) and treated with 1 μ M and 10 μ M of
483 inhibitors. The plate was centrifuged for 30 min at 32°C and 450 g. 48h after infection and
484 treatment, cells were fixed with PFA and the infected-GFP-positive cells were counted using a
485 Citation 5 Cell Imaging Multi-Mode Reader from BioTek.

486

487 **Implantation of HuARLT-rKSHV cells in mice**

488 HuARLT-rKSHV cells were transplanted to mice as described before (89). 1.2×10^6 cells were
489 seeded into each well of AggreWell™400 (27945, Stemcell Technologies), centrifuged for 3
490 minutes at 100 g and cultivated for 3 days at 37°C. 400 spheroids were used for each matrigel
491 implant containing 0,2% methylcellulose (M0512, Sigma), 3 mg/ml fibrinogen (341576,
492 Calbiochem), EGM media (CC-3124, Lonza), 10 μ g/ml FGF (100-18B-250, PeproTech), 0,5 μ g/ml
493 VEGF (RCPG246, Randox), 1 U/l thrombin (605190-100U, Merck Millipore) and 300 μ l of
494 Matrigel HC, growth factor reduced (354263, Corning). The mixture was injected
495 subcutaneously into Rag2-/- γ c-/- mice. Starting from day 1 after implantation the mice were
496 treated with dasatinib (30 mg/kg), ibrutinib (50 mg/kg), imatinib (100 mg/kg) or ponatinib (30
497 mg/kg) by oral gavage 6 times a week. Rapamycin (2 mg/kg) was injected intraperitoneally 3

498 times a week. After 4 weeks of treatment, matrigel implants were extracted and fixed with 4%
499 formalin. Diameters of the lesions were measured on sections stained for human Vimentin.
500 Animal experiments were performed in accordance with the local authorities
501 (permission/licence number #33.19-42502-04-17/2480).

502

503 **Statistical tests**

504 One-way ANOVA followed by Dunnett's multiple comparisons test and Unpaired t-Test were
505 performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla
506 California USA, www.graphpad.com).

507

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509 GB and TFS conceived the study and wrote the manuscript. DW and TD planned the animal
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519

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- 820
- 821

822 **Figure 1: TK autophosphorylation is not essential for KSHV lytic replication in tissue culture**

823 (A) Graphical representation of the TK genes from the α -herpesviruses HSV-1, HSV-2 and VZV,

824 and γ -herpesviruses EBV, KSHV, MHV-68. Phosphorylated tyrosine residues Y₆₅, Y₈₅, Y₁₂₀ and

825 glycine residues G₂₆₀, G₂₆₃, G₂₆₅ in the ATP binding pocket of KSHV TK are indicated. (B) GFP-TK

826 was overexpressed by transient transfection into HEK293 cells and immunoprecipitated 48h

827 later using a GFP antibody. Phosphorylation of GFP-TK and total GFP-TK were estimated by

828 immunoblot using a phospho-tyrosine antibody and GFP antibody, respectively. (C) GFP or GFP-

829 TK WT, 3GV or 3YF (see text) were overexpressed by transient transfection in HEK293 cells.

830 Total lysates were analysed 48h after transfection by immunoblot using antibodies to

831 phosphorylated tyrosine residues or to GFP. (D) iSLK cells stably transfected with either KSHV

832 BAC16 WT or the ORF21/TK ATP-binding pocket mutant KSHV BAC16 3GV (see text) were

833 induced with SB (1 mM) and Doxycycline (1 μ g/mL) to trigger lytic replication. Non-reactivated

834 cells were used as control. 48h after induction, cells were lysed, immunoprecipitated using

835 beads coupled to an antibody against phosphorylated tyrosine residues and analysed by

836 immunoblot using antibodies against phosphorylated tyrosine (pY) and β -actin. A

837 representative experiment is shown. Similar results were obtained with three independently

838 generated sets of iSLK populations that had been stably transfected with KSHV-wt and KSHV-

839 ORF21_3GV bacmids. (E) 48h after induction, iSLK/KSHVwt and iSLK/KSHV-ORF21_3GV cells

840 were lysed and analysed by immunoblot using antibodies against LANA, ORF45, K-bZIP and β -

841 actin. (F) Supernatants from reactivated and non-reactivated iSLK cells (E) were used to infect

842 HEK293. Infected-GFP-positive cells were counted 48h after infection.

843

844 **Figure 2: ORF21 is required to activate brivudine and zidovudine**

845 (A and B) iSLK cells stably transfected with KSHVwt (WT) or KSHV-ORF21_3GV mutant (3GV)
846 were induced with SB (1 mM) and Doxycycline (1 µg/mL) and treated with 0.01 µM up to 10 µM
847 (1/2 dilutions) of brivudine (A) or zidovudine (B). DMSO treated non-reactivated and
848 reactivated cells were used as control. Supernatants from inhibitor-treated, reactivated and
849 non-reactivated iSLK cells were used to infect HEK293. Infected-GFP-positive cells were counted
850 48h after infection. (C and D) MTT assay on iSLK was performed 48h after treatment with
851 inhibitors and reactivation.

852

853 **Figure 3: Autophosphorylation of TK in transfected cells can be inhibited by FDA-approved**

854 **kinase inhibitors**

855 Plasmids coding for GFP-TK or its empty vector (EV) were transfected in HEK293 cells. 24h after
856 transfection, cells were treated for 24 additional hours with 1 µM of FDA-approved inhibitors.
857 Total lysates were analysed by immunoblot using antibodies to phosphorylated tyrosine
858 residues or to GFP. Total GFP signal was used to normalize the ratio of phosphorylated forms of
859 TK to total TK. The complete name of the inhibitors can be found in Table 1.

860

861 **Figure 4: FDA-approved kinase inhibitors inhibit autophosphorylation of immunoprecipitated**

862 **TK**

863 (A) GFP-TK was overexpressed in HEK293 cells and immunoprecipitated 48h later using a GFP
864 antibody prior to an *in vitro* kinase assay in the presence or absence of ATP and 20 µM FDA-
865 approved inhibitors. The level of phosphorylated and total TK was estimated by immunoblot,

866 using antibodies to phosphorylated tyrosine residues and GFP, respectively, and quantification
867 on a LiCor instrument. The ratio of phosphorylated GFP-TK to total GFP in the presence of
868 inhibitor in comparison to DMSO control was calculated and listed underneath the
869 corresponding immunoblots. **(B)** Similar experiment as (A) with 10, 5, 2.5, 1.25, 0.625 and 0.313
870 μ M of bosutinib, dasatinib, ibrutinib, imatinib, ponatinib and 10, 5, 2.5, 1.25 μ M of trametinib.

871

872 **Figure 5: Inhibition of TK autophosphorylation and virus production by kinase inhibitors in**
873 **KSHV-infected cells**

874 **(A-D)** iSLK cells stably transfected with KSHV BAC16 WT were induced with SB (1 mM) and
875 Doxycycline (1 μ g/mL) and treated with 5 or 10 μ M of dasatinib **(A)**, bosutinib **(B)** or ponatinib
876 **(C)**. Non-reactivated cells were used as control. 48h after induction, cells were lysed,
877 immunoprecipitated using beads coupled to an antibody against phosphorylated tyrosine
878 residues and analysed by immunoblot using antibodies against phosphorylated tyrosine
879 residues (pY) and β -actin. **(D)** Supernatants from inhibitor-treated, reactivated and non-
880 reactivated iSLK cells were used to infect HEK293. Infected-GFP-positive cells were counted 48h
881 after infection. Unpaired t-Test was performed, *** p-value < 0.001. **(E)** Dasatinib treatment of
882 cells infected with KSHVwt or the KSHV3GV mutant. iSLK cells stably transfected with KSHVwt
883 (WT) or KSHV-ORF21_3GV mutant (3GV) were induced with SB (1 mM) and Doxycycline (1
884 μ g/mL) and treated with 0.01 μ M up to 10 μ M (1/2 dilutions) of Dasatinib. DMSO treated non-
885 reactivated and reactivated cells were used as control. Supernatants from inhibitor-treated,
886 reactivated and non-reactivated iSLK cells were used to infect HEK293. Infected-GFP-positive
887 cells were counted 48h after infection.

888

Figure 6: Inhibition of KSHV progeny production by FDA-approved tyrosine kinase inhibitors

BJAB-rKSHV.219 (A), HuARLT-rKSHV.219 (B) and Vero-rKSHV.219 (C) were used to estimate the inhibitory effect of several FDA-approved kinase inhibitors on KSHV lytic replication. Supernatants from reactivated, inhibitor-treated cells were used to infect HEK293 cells and the GFP signal in HEK293 cells measured by luminometer 48 h after infection. Data obtained from treatments with (A) 0.3 μ M, 1 μ M, 3 μ M, 10 μ M; (B and C) 0.1 μ M, 1 μ M, 10 μ M of the mentioned compounds were normalized to DMSO. Background signal was estimated by the signal obtained from uninduced cells (UI). Foscarnet (Fos) was used as a positive control at 200 μ M. Cell toxicity was assessed by MTT assay (BJAB-rKSHV.219) or DAPI staining (HuARLT-rKSHV.219, Vero-rKSHV.219). The complete name of the inhibitors can be found in Table 1. Bars and error bars represent mean $\pm$ SD accorss quadruplicate (A), triplicate (B) and duplicate of triplicate (C).

901

Figure 7: Inhibition of KSHV early protein expression by FDA-approved tyrosine kinase inhibitors

(A) BJAB-rKSHV.219 were treated with 1 μ M of inhibitors and triggered for reactivation (α -IgM) for 48h prior to lysis and analysis by immunoblot with antibodies to the early KSHV proteins K-bZIP and ORF45. The complete name of the inhibitors can be found in Table 1. (B) HEK293 were infected with rKSHV.219 (MOI 1) and treated with 1 μ M or 10 μ M of inhibitors at the time of infection. Infected-GFP-positive cells were counted 48h after infection.

909

910 **Figure 8: Inhibition of KSHV-induced tumorigenesis by FDA-approved kinase inhibitors**

911 **(A)** After implantation of HuARLT-rKSHV spheroids in mice, animals were treated *via* oral

912 gavage 6 times a week with dasatinib, ibrutinib, imatinib and ponatinib at respective

913 concentration of 30 mg/kg, 50 mg/kg, 100 mg/kg and 30 mg/kg. Rapamycin (2 mg/kg) was

914 injected 3 times a week. Four week later, the lesion diameter was estimated. One-way ANOVA

915 followed by Dunnetts' multiple comparison was performed, * p-value < 0.05. **(B)** Representative

916 pictures of lesion.

917

Table 1: List of FDA-approved kinase inhibitors used in this study^a

Agent		Drug name	Sponsor	FDA approved	Kinase inh.	Primary targets
Afatinib	(Gilotrif)	Tovok	Boehringer Ingelheim	Jul. 2013	Y	EGFR, ErbB2/4
Axitinib	(AG013736)	Inlyta	Pfizer	Jan. 2012	Y	VEGFRs, c-Kit, PDGFR β
Bosutinib	(SKI-606)	Bosulif	Wyeth	Sep. 2012	Y	BCR-Abl, Src, Lyn, Hck
Cabozantinib	(XL184)	Cometriq	Exelixis	Nov. 2012	Y	VEGFRs, c-Met, Ret, Kit, TrkB, Flt3, Tie2, Axl
Ceritinib	(LDK378)	Zykadia	Novartis	Apr. 2014	Y	ALK, IGF-1R, InsR, ROS1
Crizotinib	(PF-02341066)	Xalkori	Pfizer	Aug. 2011	Y	ALK, c-Met (HGFR), ROS1, MST1R
Dabrafenib	(6964)	Tafinlar	GlaxoSmithKline	May 2013	S/T	B-Raf
Dasatinib	(BM-354825)	Sprycel	Bristol-Myers Squibb	Jun. 2006	Y	BCR-Abl, Src, Lck, Lyn, Yes, Fyn, c-Kit, EphA2, PDGFR β
Erlotinib	(OSI-744)	Tarceva	(OSI)pharmaceuticals	Nov. 2004	Y	EGFRs
Gefitinib	(ZD1839)	Iressa	AstraZeneca	May 2003	Y	EGFRs, PDGFR
Ibrutinib	(PCI-32765)	Imbruvica	Pharmacyclics	Nov. 2013	Y	BTk
Imatinib	(STI571)	Gleevec	Novartis	May 2001	Y	BCR-Abl, c-Kit, PDGFR
Lapatinib	(GW2016)	Tykerb	GlaxoSmithKline	Mar. 2007	Y	EGFR, ErbB2
Lenvatinib	(E7080)	Lenvima	Eisai	Feb. 2015	Y	VEGFRs, PDGFRs, FGFRs, Kit, RET
Nilotinib	(AMN107)	Tasigna	Novartis	Oct. 2007	Y	BCR-Abl, PDGFR, Kit, DDR1
Nintedanib	(BIBF1120)	Ofev	Boehringer Ingelheim	Oct. 2014	Y	VEGFRs, FGFRs, PDGFRs, Lck
Palbocicnib	(PD-0332991)	Ibrance	Pfizer	Feb. 2015	S/T	CDK4, CDK6
Pazopanib	(GW-786034)	Votrient	GlaxoSmithKline	Oct. 2009	Y	VEGFRs, PDGFRs, FGDRs, c-Kit, Lck, Fms, Itk
Ponatinib	(AP24534)	Iclusig	Ariad	Dec. 2012	Y	BCR-Abl, PDGFRs, VEGFRs, FGFRs, Src, EphR, Kit, RET, Tie2, Flt3
Regorafenib	(BAY 73-4506)	Stivarga	Bayer	Sep. 2012	Y/S/T	VEGFRs, PDGFRs, Kit, RET, Raf-1, Tie2, EphA2
Ruxolitinib	(INC424)	Jakafi	Incyte	Nov. 2011	Y	JAK1/2
Sorafenib	(BAY 43-9006)	Nexavar	Bayer	Dec. 2005	Y/S/T	VEGFRs, PDGFRs, B-Raf, Kit, Flt3, RET, CDK8
Sunitinib	(SU11248)	Sutent	Pfizer	Jan. 2006	Y	VEGFRs, PDGFRs, c-Kit, RET, Flt3
Tofacitinib	(CP-690550)	Xeljanz	Pfizer	Nov. 2012	Y	JAK3
Trametinib	(6495)	Mekinist	GlaxoSmithKline	May 2013	S/T	MEK1/2

Vandetanib	(ZD6474)	Caprelsa	AstraZeneca	Apr. 2011	Y	VEGFRs, EGFRs, RET, Tie2, Brk, EphR
Vemurafenib	(PLX4043)	Zelboraf	Roche	Aug. 2011	S/T	Raf

^aFDA-approved small-molecule kinase inhibitors. More details may be found in reference 97.

A

B