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Lu Huang, Mengtian Yang, Yan Yuan, Xiaojuan Li, Ersheng Kuang

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Niclosamide Inhibits Lytic Replication of Epstein-Barr Virus by Disrupting mTOR Activation

(Running title: Niclosamide Inhibits EBV Lytic Replication)

Lu Huang[a], Mengtian Yang[a], Yan Yuan[a], Xiaojuan Li[a,]*, Ersheng Kuang[a,b,]*

[a] Institute of Human Virology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, China;

[b] Key Laboratory of Tropical Disease Control (Sun Yat-Sen University), Ministry of Education, Guangzhou, China.

*Corresponding author: Zhongshan School of Medicine, Sun Yat-Sen University, No. 74, Zhongshan 2nd Road, Guangzhou 510080, China. E-mail: kuangersh@mail.sysu.edu.cn (E. Kuang), lixjuan3@mail.sysu.edu.cn (X.Li).

Abstract

Infection with the oncogenic γ-herpesviruses Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) cause several severe malignancies in humans. Inhibition of the lytic replication of EBV and KSHV eliminates the reservoir of persistent infection and transmission, consequently preventing the occurrence of diseases from the sources of infection. Antiviral drugs are limited in controlling these viral infectious diseases. Here, we demonstrate that niclosamide, an old anthelmintic drug, inhibits mTOR activation during EBV lytic replication. Consequently, niclosamide effectively suppresses EBV lytic gene expression, viral DNA lytic replication and virion production in EBV-infected lymphoma cells and epithelial cells. Niclosamide exhibits cytotoxicity toward lymphoma cells and induces irreversible cell cycle arrest in lytically EBV-infected cells. The ectopic overexpression of mTOR reverses the inhibition of niclosamide in EBV lytic replication. Similarly, niclosamide inhibits KSHV lytic replication. Thus, we conclude that niclosamide is a promising candidate for chemotherapy against the acute occurrence and transmission of infectious diseases of oncogenic γ-herpesviruses.

1. Introduction

Two natural human oncogenic γ-herpesviruses, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), cause several types of severe malignancies (Ganem, 2010; Kutok and Wang, 2006). These viruses have two alternative lifecycles after their DNA genomes enter into the cellular nucleus: default latency and a small portion of lytic replication. Lytic replication provides a reservoir of infectious virion particles for expansion and transmission (Ganem, 2010; Kenney and Mertz, 2014). Thus, the blockade of lytic replication could effectively prevent the incidence of infection and diseases from their sources of infection. Although there are many antiviral drugs available (Siakallis et al., 2009; Skorenski and Sienczyk, 2014), few have been assessed in treating acute infection and lytic replication of these viruses.

Homologous EBV and KSHV share a high similarity of their viral DNA genomes and viral gene products (Damania, 2004; Nicholas, 2000). Consequently, these viruses employ a variety of common cellular pathways to facilitate their infection, replication and maintenance of viral genomes as well as tumorigenesis (Collins and Medveczky, 2002; Damania and Jung, 2001; de Oliveira et al., 2010; Filippakis et al., 2010; Hayward et al., 2006; Noguchi et al., 2007; Stevenson, 2004). Disruption of these pathways by inhibitors mostly leads to the inhibition of their infection and further pathogenesis; however, therapeutic clinical applications remain unavailable.

Niclosamide is one of the World Health Organization's essential medicines and is classified as an effective anthelmintic drug to treat worm infections, especially tapeworm infections (Craig and Ito, 2007). Niclosamide is also effective against

62   intractable drug-resistant bacterial infections (Costabile et al., 2015; de Carvalho et

63   al., 2011; Imperi et al., 2013; Rajamuthiah et al., 2015). As niclosamide inhibits

64   mTORC1 signaling through disruption of cellular pH homeostasis (Balgi et al., 2009;

65   Fonseca et al., 2012) and lysosome inhibition-induced Rag-mTORC1 signaling (Li et

66   al., 2013), it can be used as a preclinical inducer of autophagy. Moreover,

67   niclosamide uncouples mitochondrial respiration and disrupts cellular metabolism,

68   which provides a potential approach for treating type 2 diabetes (Tao et al., 2014).

69   As a multi-targeted inhibitor that simultaneously down-regulates the Wnt, mTOR,

70   STAT3 and NFκB pathways, emerging studies have revealed that niclosamide is a

71   potential candidate for chemotherapy of several malignant tumors (Li et al., 2014b).

72   These signaling cascades are also employed by a variety of viruses for infection and

73   replication; therefore, niclosamide shows promise as a wide-spectrum antiviral drug.

74   The inhibitory effects of niclosamide have been demonstrated in HCV and SARS

75   replication (Stachulski et al., 2011; Wu et al., 2004). Notably, a recent study showed

76   that niclosamide acts as a broad entry inhibitor for pH-dependent respiratory viruses,

77   including influenza virus and human rhinoviruses, by targeting acidic endosomes as a

78   proton carrier (Jurgeit et al., 2012). However, the therapeutic potential of

79   niclosamide in infection and diseases of DNA viruses remains to be documented.

80   In the present study, we reveal that niclosamide inhibits EBV and KSHV lytic

81   replication and causes irreversible cell cycle arrest in lytically EBV-infected B cells.

82   The effective inhibitory concentration of niclosamide in EBV lytic replication is much

83   lower than the cytotoxic dose in normal cells, indicating the therapeutic potential of

84   niclosamide as an antiviral drug against acute infection and diseases of human

85   γ-herpesviruses.

2. Methods and Materials

2.1. Cells, chemicals and antibodies. The EBV-negative and KSHV-negative lymphoma cell line BJAB, the EBV-positive lymphoma cell lines P3HR-1 and Akata(+), the EBV-positive epithelial cell line C666-1, the HNE1 cells stably transfected with the EBV p2089 bacmid (HNE1-2089), and the KSHV-positive lymphoma cell line BCBL1 were maintained in our laboratory or were provided by Prof. Musheng Zeng (Cancer Center of Sun Yat-sen University). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). Niclosamide (Catalog number N3510), thiazolyl blue tetrazolium blue (MTT), 12-O-tetradecanoylphorbol-13-acetate (TPA) and sodium butyrate (NaB) were purchased from Sigma-Aldrich Co. (St. Louis, MO). CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assays (MTS) were purchased from Promega (Madison, WI). Anti-mTOR, p-mTOR S2448, S6K1, p-S6K1, 4EBP1, and p-4EBP1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-BZLF1 and BMRF1 antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-EBNA1 antibodies were purchased from Genetex Inc. (Irvine, CA). Anti-RTA, ORF64, K8 and LANA antibodies have been previously described (Li et al., 2015a). Amaxa® Cell Line Nucleofector® Kit V was purchased from LONZA, Switzerland. mTOR expressing plasmid was a gift from Drs. Enbo Liu and Gary G. Chiang of the Sanford-Burnham Medical Research Institute, La Jolla, CA.

2.2. Isolation and culture of peripheral blood mononuclear cells (PBMCs). Whole blood of healthy donors was purchased from the blood banks of Guangzhou Blood Center. PBMCs were isolated using Lymphocyte Separation Medium (LONZA)

109 according a standard procedure and cultured in RPMI 1640 medium containing 10%

110 FBS and antibiotics. All PBMC work was conducted according to the guidelines and

111 was approved by the medical ethics committee at Sun Yat-sen University.

112 **2.3. Western blot analysis.** Cells were collected and lysed with cell lysis buffer (50

113 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 10% glycerol, 40 mM

114 β-glycerophosphate, 30 mM sodium fluoride, 5 mM EDTA, 1× protease inhibitor

115 cocktail (Roche), 1 mM sodium orthovanadate). After shaking at 4℃ for 30 min, the

116 whole cell lysates were centrifuged at 13,000 rpm for 10 min at 4℃. The whole cell

117 extracts were resolved by SDS-PAGE (40 μg protein/each lane) and transferred to

118 nitrocellulose membranes. The membranes were blocked with 5% not-fat dry milk in

119 PBS plus 0.1% Tween-20 and then incubated with primary antibodies overnight at

120 4℃. Anti-Infrared Dye 800 or Dye 680 or HRP-labeled anti-rabbit or anti-mouse IgG

121 were used as the secondary antibodies. The images were visualized using the LI-COR

122 Odyssey system or detected using the enhanced chemiluminescence system (Bio-rad)

123 followed by X-ray film exposure. All immunoblots were repeated at least twice, and

124 representative images are shown.

125 **2.4. Induction of viral lytic replication.** P3HR-1 and Akata(+) cells were induced by

126 20 ng/mL TPA plus 3 mM NaB and 0.8% (v/v) goat anti-human IgG (Shuangliu

127 Zhenglong Biochem Lab, China), respectively. HNE1-2089 cells were induced by 20

128 ng/mL TPA plus 3 mM NaB or transfected with a BZLF1-expressing plasmid. C666-1

129 cells were transfected with a BZLF1-expressing plasmid. BCBL1 cells were treated

130 with 20 ng/mL TPA to induce KSHV lytic replication.

131 **2.5. Detection of viral genomic DNA and virion production.** The cells were collected

132 at 48 h post-treatment, and the intracellular viral genomic DNA was extracted from

133 cell pellets using a Magen Hipure Tissue DNA mini Kit (Magen, China). To extract EBV

134 virions, cell culture media were collected at 5 days post-treatment and passed

135 through 0.45 μm filters to remove the cell debris. Then, the supernatant was

136 digested with DNaseI at 37℃ for 1 h, and the reaction was stopped by EDTA at 70℃

137 for 10 min followed by proteinase K digestion. Then, virion DNA was extracted

138 following a standard procedure. The amounts of intracellular viral DNA and virion

139 DNA were determined by real-time PCR. The real-time PCR primer pairs were as

140 follows: EBNA1, forward, CATTGAGTCGTCTCCCCTTTGGAAT, reverse,

141 TCATAACAAGGTCCTTAATCGCATC; LANA, forward, CGCGAATACCGCTATGTACTCA,

142 reverse, GGAACGCGCCTCATACGA; GAPDH, forward, ACATCATCCCTGCCTCTAC,

143 reverse, TCAAAGGTGGAGGAGTGG. The amounts of intracellular genomic viral DNA

144 and extracellular virion DNA were normalized to cellular genomic GAPDH and the

145 cell number (per 10^5 cells/ml), respectively. The mean ± standard deviation and half

146 inhibitory concentration (IC50) values were calculated using GraphPad software from

147 three independent experiments performed in triplicate, and the significance was set

148 to $p < 0.01$ after the data were analyzed using Student's t-test with a two-sample

149 unequal variance and two-tailed distribution.

150 **2.6. Detection of cell viability and proliferation.** P3HR-1, Akata(+), BJAB and PBMCs

151 were left untreated or treated with different concentrations of niclosamide for 48 h,

152 and then the cells were stained by trypan blue. The living and dead cells were

153 analyzed using Countstar® automated cell counter. The curve of cell viability was

154 determined according to the percentage of living cells. After EBV latently or lytically

155 infected P3HR-1 and EBV-negative BJAB cells were left untreated or treated with

156 niclosamide for 48 h, cell proliferation was detected using CellTiter 96® AQ$_{ueous}$

157 One-Solution cell proliferation assay (MTS). The One-Solution reagent was added to

158 the 96-well plates, and the absorbance at 490 nm after 4 h of incubation was

159 measured. The curve of cell proliferation was generated according to the ratio of

160 OD$_{490}$ (sample)/OD$_{490}$ (control). The 50% cytotoxic concentration (CC50) was

161 calculated from three independent experiments performed in triplicate using

162 GraphPad Prism software. HNE1-2089 cells were left untreated or treated with

163 niclosamide for 48 h, and the cell proliferation was detected using an MTT assay.

164 Briefly, 20 μl of 5 mg/ml MTT per well was added and incubated for 4 h, and then

165 the media were removed carefully, and 150 μl of DMSO per well was added. After

166 the cell plates were shaken for 15 min, the absorbance at 490 nm was recorded, and

167 the CC50 was calculated as described above.

168 **2.7. Measurement of cell cycle.** Two days after niclosamide treatment, cells were

169 collected and fixed with 70% ethanol at 4℃ overnight. Then cells were washed with

170 cold PBS, treated with 100 ng/ml RNaseI and 0.1% Triton-X100 at 37℃ for 30 min,

171 followed by staining with 50 μg/ml propidium iodide (PI) and measured by BD

172 LSRFortessa FACS system (BD Bioscience). The cell cycle was analyzed and quantified

173 using FlowJo software.

174 **2.8. Nucleofection.** P3HR-1 cells were passaged one day prior to nucleofection, and

175 then 1 x 10^7 cells per sample were washed with PBS once and resuspended carefully

176 in 100 μl of Nucleofector® Solution at room temperature. DNA (5 μg) was mixed with

177 100 μl of cell suspension, and the mixtures were nucleofected using preliminary

178 Nucleofector® Program C-009. After transfection, 500 μl of pre-warmed culture

179 medium (RPMI 1640 and 10% fetal bovine serum, without antibiotics) was added to

180 the cuvette immediately, and the cells were gently transferred into a T25 flask with

181 fresh medium.

182

3. Results

3.1. Niclosamide inhibits mTOR activation during EBV lytic replication

mTOR activation plays an important role in EBV-related cancer, but less is known

about mTOR in EBV lytic replication. Here, we detected the phosphorylation of

mTOR and the substrates S6K1 and 4EBP1 during EBV lytic replication. When

EBV-positive P3HR-1 cells and EBV-negative BJAB cells were induced with TPA plus

NaB, the phosphorylation of mTOR at Ser2448 and its substrates S6K1 and 4EBP1

were increased in EBV lytically infected P3HR-1 cells, whereas little difference was

observed in BJAB cells (Figure 1A). The expression levels of these proteins were

unaffected. Therefore, we conclude that the mTOR pathway was activated during

EBV lytic replication.

Niclosamide is a novel mTOR inhibitor; we determined whether niclosamide

inhibited mTOR activation during EBV lytic replication. After TPA plus NaB induction,

EBV lytically infected P3HR-1 cells were treated with different amounts of

niclosamide. Niclosamide inhibited the phosphorylation of mTOR and two substrates,

S6K1 and 4EBP1, during EBV lytic replication in a dose-dependent manner, and ≥ 0.5

μM niclosamide almost had the maximal inhibition (Figure 1B), but exerted no effect

on their expression. Similar inhibition was observed in Akata(+) cells after anti-IgG

induction (Figure 1C). These results suggest that niclosamide suppresses the

activation of mTOR and the substrates S6K1 and 4EBP1 during EBV lytic replication.

3.2. Niclosamide inhibits EBV lytic replication

Next we detected whether niclosamide inhibited EBV lytic replication. EBV lytically

206 infected P3HR-1 cells were treated with different amounts of niclosamide. The levels

207 of the lytic proteins BZLF1 and BMRF1 were dramatically decreased by niclosamide

208 in a dose-dependent manner, and ≥ 0.5 µM niclosamide almost exhibited complete

209 inhibition but did not affect the level of the EBV latent protein EBNA1 (Figure 2A).

210 Further we examined the effects of niclosamide on EBV DNA lytic replication and

211 virion production. Niclosamide significantly inhibited EBV intracellular DNA

212 replication and virion production (Figure 2B-C). The 50% inhibitory concentration

213 (IC50) values of intracellular viral genomic DNA replication and extracellular virion

214 yield were 0.13 µM and 0.092 µM, respectively, and 0.5 µM exhibited >95%

215 inhibition of both viral DNA replication and virion production. Similar inhibition of

216 niclosamide was observed in Akata(+) cells after anti-IgG induction. The expression

217 of lytic genes BZLF1 and BMRF1 but not latent gene EBNA1 were greatly decreased

218 by niclosamide at ≥1 µM; the IC50 values of viral DNA replication and virion yield

219 were 0.20 µM and 0.18 µM, respectively, appropriately 80% inhibition of viral DNA

220 replication because of high spontaneous lytic replication (Figure 2E) and >95%

221 inhibition of virion production occurred at ≥1 µM (Figure 2F). Furthermore, a

222 well-known mTOR inhibitor, rapamycin, was assessed as a control for EBV lytic

223 replication in lymphoma cells. It slightly inhibited the expression of the EBV lytic

224 genes BZLF1 and BMRF1, as well as virion production in P3HR-1 cells following the

225 induction of TPA plus NaB (Figure 2G-H); however, it did not exhibit any inhibition in

226 Akata(+) cells after anti-IgG induction (Figure 2I-J). These results suggest that

227 niclosamide effectively inhibits EBV lytic replication in lymphoma cells.

228   Studies have shown that the mTOR inhibitor rapamycin is not capable of inhibiting

229 EBV lytic replication in epithelial cells (Adamson et al., 2014); we determined

230 whether niclosamide suppressed EBV lytic replication in nasopharyngeal carcinoma

231 (NPC) epithelial cells. EBV p2089 bacmid-harboring HNE1 cells were induced by TPA

232 plus NaB for lytic replication, and then different amounts of niclosamide were added

233 and incubated. The expression of the lytic genes BZLF1 and BMRF1 were decreased

234 following the increase of niclosamide, whereas the expression of the latent gene

235 EBNA1 was barely reduced (Figure 3A). The IC50 of virion production was 0.15 μM,

236 with more than 90% inhibition at ≥1 μM (Figure 3B). Further ectopic BZLF1

237 expression was introduced into HNE1-2089 cells to initiate lytic replication.

238 Niclosamide did not affect the ectopic GFP-BZLF1 expression, whereas it greatly

239 inhibited the expression of the lytic gene BMRF1 and endogenous BZLF1,

240 intracellular viral DNA replication and virion production (Figure 3C-E). This indicates

241 that niclosamide suppresses BZLF1-mediated EBV lytic replication in epithelial cells.

242 As expected, neither viral gene expression nor virion production was inhibited by

243 rapamycin in these epithelial cells (Figure 3F-G). Similarly, when the natural

244 EBV-positive NPC-derived epithelial cells C666-1 were treated with different

245 concentrations of niclosamide following ectopic GFP-BZLF1 expressing transfection,

246 inhibition of EBV lytic gene expression and lytic replication was observed, with an

247 IC50 of 0.07 μM for virion production (Figure 3H-I). Therefore, we conclude that

248 niclosamide inhibits EBV lytic replication in NPC epithelial cells.

249

250 **3.3. Niclosamide induces cell cycle arrest in EBV-infected B cells**

251 Further we detected the cytotoxicity of niclosamide in lymphoma cells and normal

252   cells. We found that lymphoma cells were sensitive to niclosamide. Niclosamide

253   exerted a similar curve of cytotoxicity in P3HR-1 and BJAB cells, the 50% cytotoxic

254   concentration CC50 values were 1.06 μM and 1.49 μM, respectively, and the CC50 in

255   Akata(+) cells was 3.83 μM (Figure 4A). However, normal human PBMCs were

256   insensitive to niclosamide at the low concentration, the CC50 was >80 μM (Figure

257   4B). Cell proliferation was examined in P3HR-1 and BJAB cells that were left

258   untreated or after TPA/NaB induction and niclosamide treatment. Niclosamide

259   exhibited similar inhibition curves and IC50 values of cell proliferation in BJAB cells

260   regardless of TPA/NaB induction, whereas the inhibition was slightly stronger in lytic

261   cells than that in latent cells (Figure 4C-D). Additionally, the CC50 of cell viability was

262   6.03 μM in HNE1-2089 epithelial cells (Figure 3I) and 0.25 μM in C666-1 cells (Figure

263   3J), which was far lower than that in PBMCs. The difference of cell viability between

264   tumor cells and PBMCs indicates that niclosamide effectively suppresses lymphoma

265   cells and NPC epithelial cells compared with normal cells.

266   To further define the inhibitory role of niclosamide in EBV-infected B cells, we

267   detected the cell cycle of P3HR-1 and BJAB cells under niclosamide treatment. We

268   found that niclosamide induced cell cycle arrest in EBV lytically infected P3HR-1 cells;

269   the percentage of S phase decreased from 28% to 7% in the presence of niclosamide

270   (≥5 μM) (Figure 4D, panel c), whereas niclosamide exerted a minor effect on cell

271   cycle arrest in BJAB cells and latently infected P3HR-1 cells (Figure 4D, panel a-b). To

272   further characterize the cell cycle arrest, niclosamide was withdrawn after 24 h

273   incubation; the inhibition of cell cycle was not reversed, and apoptotic cells were

274   observed in EBV lytically infected P3HR-1 cells after niclosamide was withdrawn at ≥

275 2 µM, whereas apoptotic cells were barely observed in the presence of niclosamide

276 (Figure 4D, panel d). Given that niclosamide caused slightly stronger inhibition in

277 lytic cells than in latent cells (Figure 4C), these results suggest that niclosamide

278 causes irreversible cell cycle arrest and subsequently cell death in EBV lytically

279 infected B cells. Then we conclude that niclosamide exhibits more effective

280 inhibition in lytically EBV-infected lymphoma cells than in latent EBV-infected and

281 uninfected lymphoma cells.

282   In current medical practice, the dose of niclosamide is 1-2 g daily in adults (oral

283 administration, ≥ 100 µM in intestine) for treating worm infections (World Health

284 Organization., 1995) and 40-200 mg/kg daily (injection, approximately 100-500 µM)

285 in anticancer experimental studies (Jin et al., 2010; King et al., 2015); both of these

286 doses are much higher than the effective inhibitory concentration for EBV lytic

287 replication and lytic EBV-infected cells. Therefore, we believe that niclosamide is an

288 effective drug against acute EBV infection in patients.

289

290 **3.4. mTOR overexpression restores niclosamide-inhibited EBV lytic replication**

291   To further determine whether the effect of niclosamide on EBV lytic replication is

292 due to inhibition of mTOR activation, we transfected an mTOR-expressing plasmid

293 into P3HR-1 cells, followed by niclosamide treatment. After induction, the

294 phosphorylation of S6K1 was restored by ectopic mTOR expression in the presence

295 of niclosamide. As result, 0.2 µM niclosamide no longer inhibited the expression of

296 the EBV lytic genes BZLF1 and BMRF1 in the cells overexpressing mTOR, whereas

297 there was inhibition in control cells (Figure 5A); the inhibition of viral DNA replication

298 and virion yield were restored from >90% inhibition in control cells to <50% in cells

299 overexpressing mTOR (Figure 5B-C), suggesting that ectopic mTOR overexpression

300 was able to rescue the inhibition of EBV lytic replication by niclosamide. Although

301 ectopic mTOR expression completely recovered S6K1 activity and the expression of

302 the lytic genes BZLF1 and BMRF1 in the presence of 0.2 µM niclosamide, it was not

303 able to completely restore EBV virion production. It is possible that niclosamide

304 limits multiple signaling pathways that are required for late lytic gene expression and

305 disrupts the intracellular proton homeostasis that are required for EBV virion

306 assembly/transportation in the acidic autophagosome/lysosome (Granato et al.,

307 2014; Jurgeit et al., 2012; Li et al., 2014b; Murata, 2014) . These results suggest that

308 niclosamide suppresses EBV lytic replication mainly by disrupting mTOR activation.

309

310 **3.5. Niclosamide inhibits KSHV lytic replication in B cells**

311 Finally, we investigated whether niclosamide exhibited universal inhibition of

312 oncogenic γ-herpesvirus. After KSHV lytic replication was induced in BCBL1 cells by

313 TPA, 0.2 µM niclosamide strongly inhibited phosphorylation of mTOR as well as two

314 substrates, 4EBP1 and S6K1 (Figure 6A top). Thus, ≥0.5 µM niclosamide dramatically

315 inhibited the expression of KSHV lytic genes (RTA, ORF64 and K8) and barely affected

316 the expression of the latent gene LANA (Figure 6A bottom). Consequently, viral DNA

317 replication was reduced following the increase in niclosamide concentration; the

318 IC50 was 0.14 µM and the concentration required for ≥80% inhibition was ≥0.5 µM

319 (Figure 6B). Similarly, virion production was suppressed by niclosamide in a

320 dose-dependent manner, with an IC50 of 0.17 µM and more than 90% inhibition at

321   ≥0.5 μM (Figure 6C). These results show that niclosamide commonly inhibits lytic

322   replication of both human γ-herpesviruses.

4. Discussion

Studies have revealed that niclosamide is a novel mTORC1 inhibitor and autophagy inducer through lowering cytoplasmic pH and modulating Rag-GTPase (Balgi et al., 2009; Fonseca et al., 2012; Li et al., 2013). In the present study, we revealed that niclosamide disrupts mTOR activation during EBV lytic replication, consequently inhibiting EBV lytic replication in both lymphoma cells and NPC epithelial cells, and inducing irreversible cell cycle arrest in lytic EBV-infected B cells. Ectopic mTOR overexpression restores the inhibition of EBV lytic replication in the presence of niclosamide. Similarly, niclosamide significantly suppresses KSHV lytic replication. Our findings suggest that niclosamide significantly suppresses EBV and KSHV lytic replication by disrupting mTOR activation and preferentially kills lytic EBV-infected cells; these findings highlight the promising therapeutic potential of this old drug for treating EBV and KSHV-related infectious diseases.

Although inhibiting mTOR activation and mTOR inhibitors have been demonstrated to effectively control KSHV-related diseases (Diaz-Ley et al., 2015; Nichols et al., 2011; Sin et al., 2007), this approach has been undefined in the chemotherapy of EBV-related diseases. The inhibition of mTOR activation exhibits an ambiguous effect on EBV lytic infection. Rapamycin decreases EBV lytic replication in lymphoma cells but does not affect the expression of latent or lytic genes in EBV-positive epithelial cell lines and EBV-associated T and NK lymphoma cells (Adamson et al., 2014; Kawada et al., 2014). Because latent EBV-infected cells exhibit active mTOR pathways that are activated by EBV-encoded LMP1 and LMP2A (Chen

346 et al., 2010; Moody et al., 2005), the inhibition of this pathway results in cell cycle

347 arrest and consequently inhibits tumor growth in EBV-associated T and NK

348 lymphoma (Kawada et al., 2014). However, the administration of rapamycin alone

349 might not be sufficient for curing these diseases (Holtan et al., 2008); novel

350 inhibitors or combined chemotherapy that have more significant efficacy are

351 required for this strategy (Furukawa et al., 2013; Wong et al., 2013). Our study

352 reveals that niclosamide inhibits EBV lytic replication in both lymphoma cells and

353 NPC epithelial cells. Furthermore, lytic EBV-infected cells are killed by niclosamide

354 through irreversible G1/S cell cycle arrest; DNA damage and DNA damage responses

355 are induced during EBV lytic replication (Gargouri et al., 2011; Ma et al., 2008;

356 Wang'ondu et al., 2015), therefore, lytic EBV infection increases susceptibility to

357 niclosamide. Our findings suggest that niclosamide might effectively prevent the

358 acute occurrence and transmission of EBV-related infectious diseases and that

359 niclosamide may be a promising therapeutic approach for EBV-associated diseases.

360

361   Studies have shown that niclosamide inhibits cell proliferation and induces

362 apoptosis and cell cycle arrest (Lee et al., 2014; Li et al., 2015c; Liao et al., 2015; Lu

363 et al., 2011; Ye et al., 2014). In lytic EBV-infected lymphoma cells, niclosamide

364 treatment caused cell cycle arrest with a slight increase in cell death, while apoptosis

365 was dramatically elevated in response to niclosamide treatment and following

366 withdrawal (Figure 4E). Presumably, both cell proliferation and apoptosis were

367 simultaneously halted in the presence of niclosamide because inhibition of the

368 mTOR pathway blocks protein synthesis (Fonseca et al., 2014). Following the

369 withdrawal of niclosamide, mTOR activation and protein synthesis were recovered,

370 and apoptotic processes were subsequently executed. Because cell cycle arrest is

371 irreversible, the pulsed niclosamide treatment might provide more effective

372 inhibition in lytic EBV-infected cells than continuous treatment.

373

374 Niclosamide simultaneously inhibits multiple cellular pathways, including the

375 Wnt, Notch and NFκB pathways (Li et al., 2014b) and acts as an inhibitor of the

376 STAT3 pathway (Ren et al., 2010). However, these mechanisms are not responsible

377 for its inhibition of EBV lytic replication. Inhibition of Notch signaling can induce the

378 EBV lytic cycle (Giunco et al., 2015) and activated Notch-2 inhibits the EBV lytic cycle

379 (Rowe et al., 2014); therefore, the disruption of Notch signaling is not related to the

380 inhibition of EBV lytic replication by niclosamide. Alternatively, BZLF1 blocks NFκB

381 phosphorylation and TNFα expression to promote optimal lytic replication (Li et al.,

382 2015b), and we found that niclosamide inhibits NFκB phosphorylation but barely

383 reduces STAT3 phosphorylation during EBV lytic replication (data not shown). Thus,

384 its inhibition of EBV lytic replication is not due to inhibition of NFκB or STAT3 activity.

385 Our results showed that mTOR overexpression completely restored the expression of

386 the lytic genes BZLF1 and BMRF1 in the presence of niclosamide, indicating that

387 niclosamide disrupts EBV IE gene expression primarily through inhibition of mTOR

388 activation (Figure 5A). However, mTOR overexpression partially but not completely

389 restored DNA replication and virion production (Figure 5B-C). Niclosamide also

390 possesses protonophoric activity (Fonseca et al., 2012; Jurgeit et al., 2012), and this

391 activity probably plays a role in its inhibition of maturation of EBV virions because

392 EBV lytic replication requires acidic autophagic vesicles/lysosomes for virion

393 assembly and transportation (Granato et al., 2014). However, we cannot exclude the

394 other possibility due to the limitations of our experiments.

395

396 EBV and KSHV are two homologues of oncogenic γ-herpesvirus that share

397 multiple pathways for their infection and pathogenesis (Damania, 2004; Nicholas,

398 2000; Noguchi et al., 2007). Our study reveals that niclosamide inhibits the lytic

399 replication of both γ-herpesviruses that require mTOR activation and induces a

400 cytotoxic effect in lymphoma cells that are infected by these viruses, suggesting that

401 niclosamide controls their infection at two levels: inhibiting viral replication and

402 inducing cell death, which provides a more effective strategy for EBV and

403 KSHV-related diseases. Niclosamide exerts an inhibitory effect on lytic replication of

404 both γ-herpesviruses and cytotoxicity at effective doses much lower than the

405 acceptable dose in patients or experimental animals, suggesting that niclosamide is a

406 promising drug for controlling the infectious diseases of both viruses.

407

408 Although niclosamide inhibits the infection and replication of certain RNA viruses

409 (Fang et al., 2013; Jurgeit et al., 2012; Wu et al., 2004), it does not affect HSV-1

410 replication because the 50% inhibition concentration of niclosamide in HSV-1

411 replication (exceeding 10 μM) closely matches the concentration of cytotoxicity

412 (Jurgeit et al., 2012). Another mTOR inhibitor, rapamycin, does not exert any

413 substantial effect on the replication of γ34.5-deleted HSV-1 (Fu et al., 2011).

414 Additionally, the inhibitory effect of niclosamide has not been observed in other

415 DNA viruses to date. In fact, mTOR inhibitors are mainly used as immunosuppressive

416 drug and anticancer agent (Li et al., 2014a; Tsang et al., 2007). Given that

417 niclosamide has exhibited a large significance in cancer therapy (Li et al., 2014b),

418 niclosamide might not exhibit broad-spectrum antiviral efficacy against

419 herpesviruses, but could potentially prevent the incidence of infectious diseases and

420 malignancies of DNA tumor viruses.

421

422 Altogether, we revealed that an old anthelmintic drug, niclosamide, suppresses

423 EBV lytic replication in lymphoma cells and epithelial cells and induces irreversible

424 cell cycle arrest in lytic EBV-infected cells, mainly through its novel function of

425 mTORC1 inhibition. Additionally, niclosamide inhibits KSHV lytic replication, a

426 homologous human γ-herpesvirus. Therefore, our findings provide a promising use

427 of this old drug for treating acute EBV and KSHV-associated infectious diseases.

428 **Acknowledgments**

433

434

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Figure legends

Figure 1. Effects of niclosamide on mTOR activation during EBV lytic replication. (A) P3HR-1 and BJAB cells were left untreated or treated with 20 ng/ml TPA plus 3 mM NaB for 0, 24 h or 48 h, and then the cells were collected. Whole cell lysates were then subjected to Western blot analysis as indicated. (B) P3HR-1 cells were induced by 20 ng/ml TPA plus 3 mM NaB for 3 h, and (C) Akata(+) cells were induced by 0.8% (v/v) anti-IgG for 3 h, and then both were followed by niclosamide treatment (NIC) at different concentrations for an additional 48 h. The cells were collected, and whole cell lysates were analyzed as indicated.

Figure 2. Effects of niclosamide on EBV lytic replication in lymphoma cells. (A-B) P3HR-1 cells were induced by 20 ng/ml TPA plus 3 mM NaB for 3 h and then treated with niclosamide at different concentrations for an additional 48 h. (A) The cells were collected, and the expression levels of EBV genes were detected by Western blot analysis. (B) P3HR-1 cells were induced and treated as described above, and the intracellular EBV genomic DNA was extracted and determined by real-time PCR. The relative levels were normalized to cellular genomic GAPDH. (C) P3HR-1 cells were induced by TPA and NaB for 3 h and then left untreated or treated with niclosamide at different concentrations for 5 days. The supernatants were collected, and EBV virion DNA was extracted and determined by real-time PCR. (D-F) Akata(+) cells were induced by anti-IgG for 3 h and then treated with niclosamide as described above. The viral gene expression (D), intracellular viral DNA (E) and virion production (F) were detected. The means ± SD and IC50 were calculated as described in Materials

604 and Methods and are shown. (G-H) P3HR-1 cells and (I-J) Akata(+) cells were induced

605 as described above and then left untreated or treated with the different

606 concentrations of rapamycin (Rapa) as indicated. Viral gene expression (G, I) and

607 virion production (H, J) were then analyzed as described above. NS, No statistical

608 difference.

609

610 **Figure 3. Effects of niclosamide on EBV lytic replication in epithelial cells.** (A-C)

611 HNE1-2089 cells were induced by 20 ng/ml TPA plus 3 mM NaB for 3 h and then

612 treated with niclosamide. (A) Two days later, the cells were collected, and the

613 expression of EBV related proteins was analyzed as indicated. (B) After niclosamide

614 treatment for 5 days, EBV virion DNA was extracted from the cell supernatant and

615 detected by real-time PCR. (C-E) HNE1-2089 cells were transfected with

616 GFP-BZLF1-expressing plasmid overnight and then left untreated or treated with 0.5

617 μM niclosamide for 48 h, and the viral gene expression (C) and the intracellular EBV

618 DNA (D) were detected, GFP-BZLF1 indicates ectopic BZLF1 expression, BZLF1

619 indicates endogenous BZLF1 expression. The EBV virion DNA level was determined

620 after niclosamide treatment for 5 days (E). (F-G) The HNE1-2089 cells were induced

621 as described above and then left untreated or treated with rapamycin as indicated.

622 Subsequently, viral gene expression (F) and virion production (G) were detected. (H-I)

623 The C666-1 cells were transfected with GFP-BZLF1 plasmid overnight and then left

624 untreated or treated with niclosamide at different concentrations (0-0.5 μM); viral

625 gene expression (H) and virion production (I) were then detected. (J, K) The cell

626 viability and CC50 of niclosamide in HNE1-2089 cells (J) and in C666-1 cells (K) were

627 examined as described in Materials and Methods.*, p<0.01.

628

629 **Figure 4. Effects of niclosamide on cell viability and cell cycle in B cells.** (A) P3HR-1,

630 Akata(+), BJAB cells and (B) PBMC cells were treated with different amounts of

631 niclosamide for 48 h, and the cells were stained by trypan blue to count the living

632 and dead cells. The curve of cell viability and CC50 were determined according to

633 the percentage of living cells. (C) P3HR-1 and (D) BJAB cells were left uninduced or

634 induced with 20 ng/ml TPA plus 3 mM NaB for 3 h and then treated with niclosamide

635 at different concentrations. After 48 h, cell proliferation was detected by CellTiter

636 96® AQ$_{ueous}$ One-Solution cell proliferation assay, and the CC50 was determined

637 according to relative cell proliferation. (E) Two days after niclosamide treatment,

638 P3HR-1 cells were fixed and stained with PI, and the cell cycle was detected using

639 FACS system. Shown are the cell cycles in BJAB cells (a), P3HR-1 cells (b), P3HR-1 cells

640 after TPA induction (c) and TPA induced-P3HR-1 cells with niclosamide withdrawn

641 after treatment for 24 h (d). Representative images are shown, and the percentages

642 of S and sub-G$_1$ phase were calculated from three independent experiments.

643

644 **Figure 5. mTOR overexpression rescues niclosamide-inhibited EBV replication.**

645 P3HR-1 cells were transfected with mTOR-expressing plasmids or with control vector.

646 After transfection for 24 h, cells were induced by TPA plus NaB for 3 h and then left

647 untreated or treated with 0.2 μM niclosamide for an additional 48 h. (A) Whole cell

648 lysates were subjected to Western blot analysis as indicated. (B) The intracellular

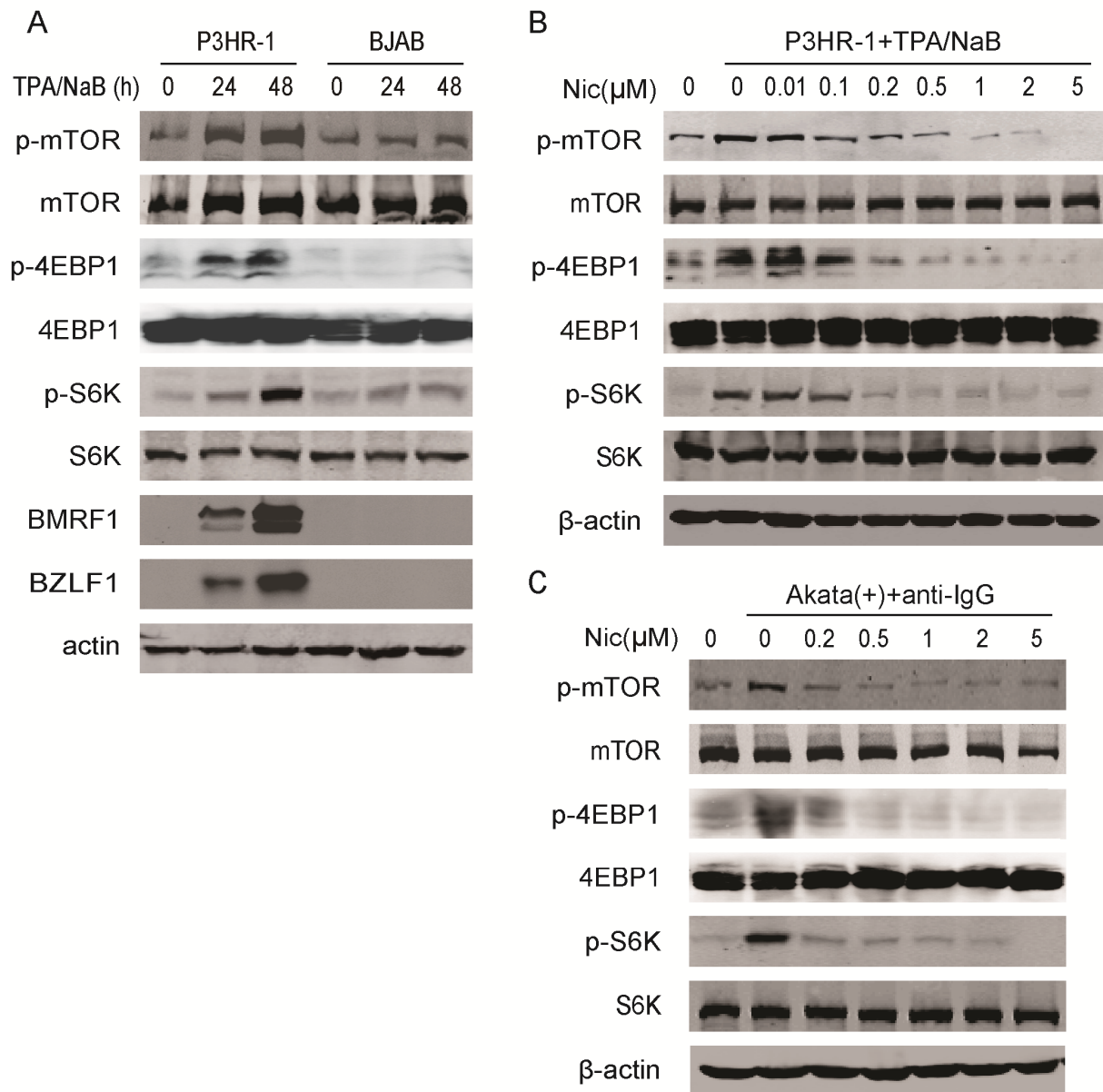649 EBV DNA was extracted and quantified by real-time PCR. (C) EBV virion DNA was

650 extracted from the supernatant and determined by real-time PCR after niclosamide

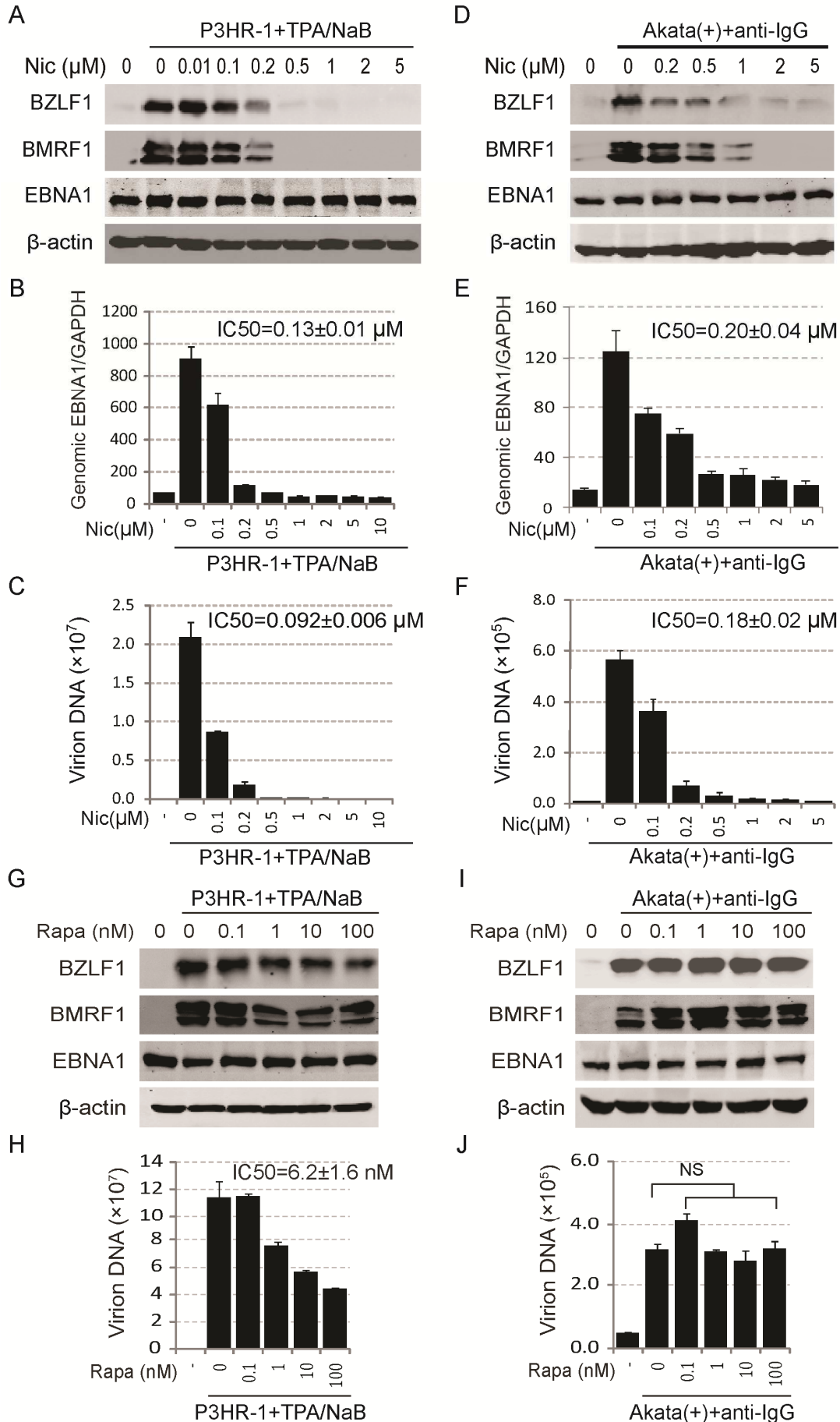651 incubation for 5 days. *, p<0.01.

652

653 **Figure 6. Effects of niclosamide on KSHV lytic replication.** (A) BCBL1 cells were

654 induced by 20 ng/ml TPA for 3 h, and then different amounts of niclosamide were

655 added and incubated for an additional 48 h. The cells were collected, and the

656 phosphorylation of mTOR and the two substrates, 4EBP1 and S6K1, and the

657 expression of KSHV genes were detected as indicated. (B) The cells were treated as

658 described above, and viral genomic DNA inside the cells was extracted and analyzed

659 by real-time PCR. (C) BCBL1 cells were induced by 20 ng/ml TPA and treated with

660 different amounts of niclosamide for 5 days. KSHV virion DNA was extracted and

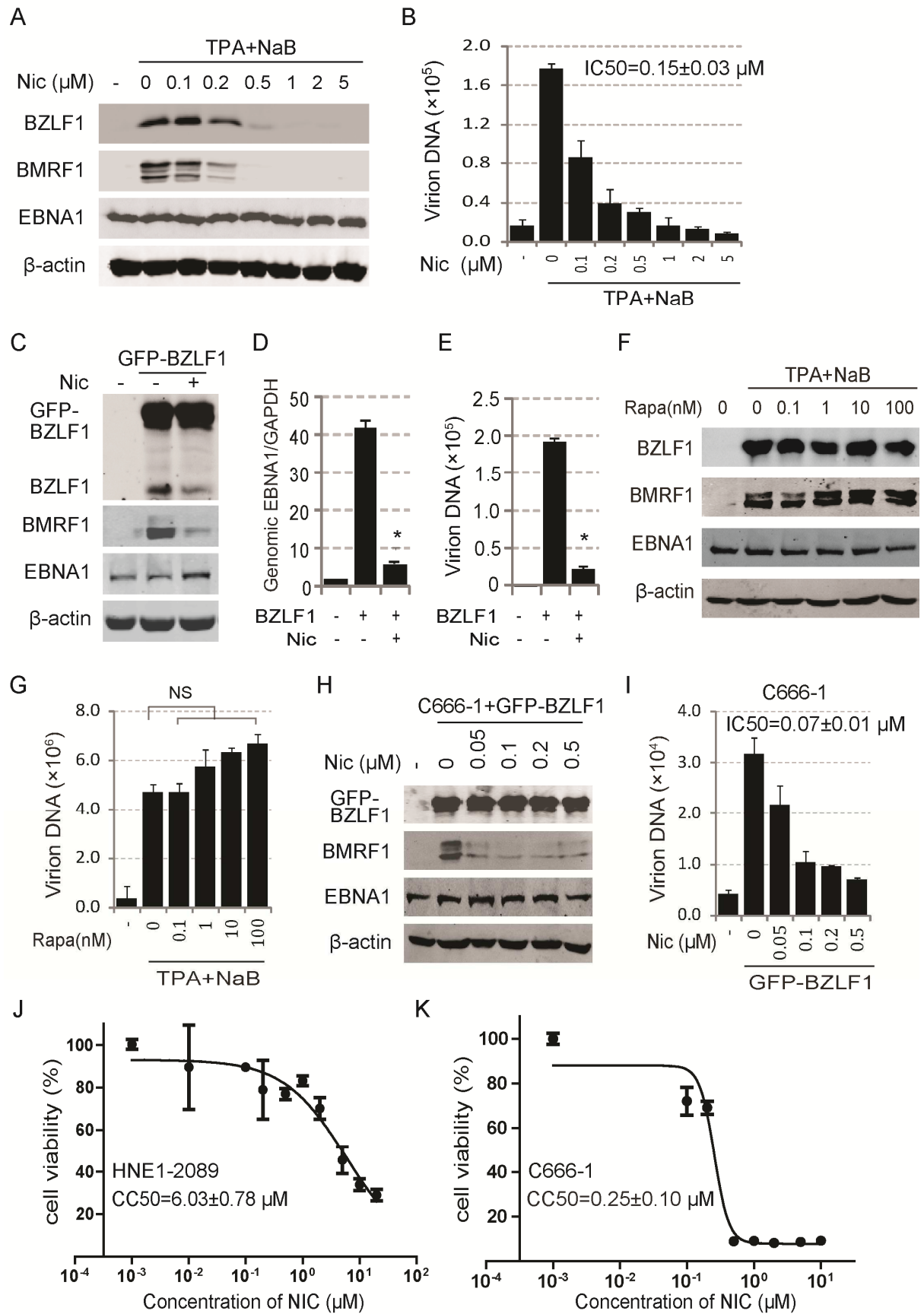661 determined by real-time PCR, and the IC50 was calculated from three independent
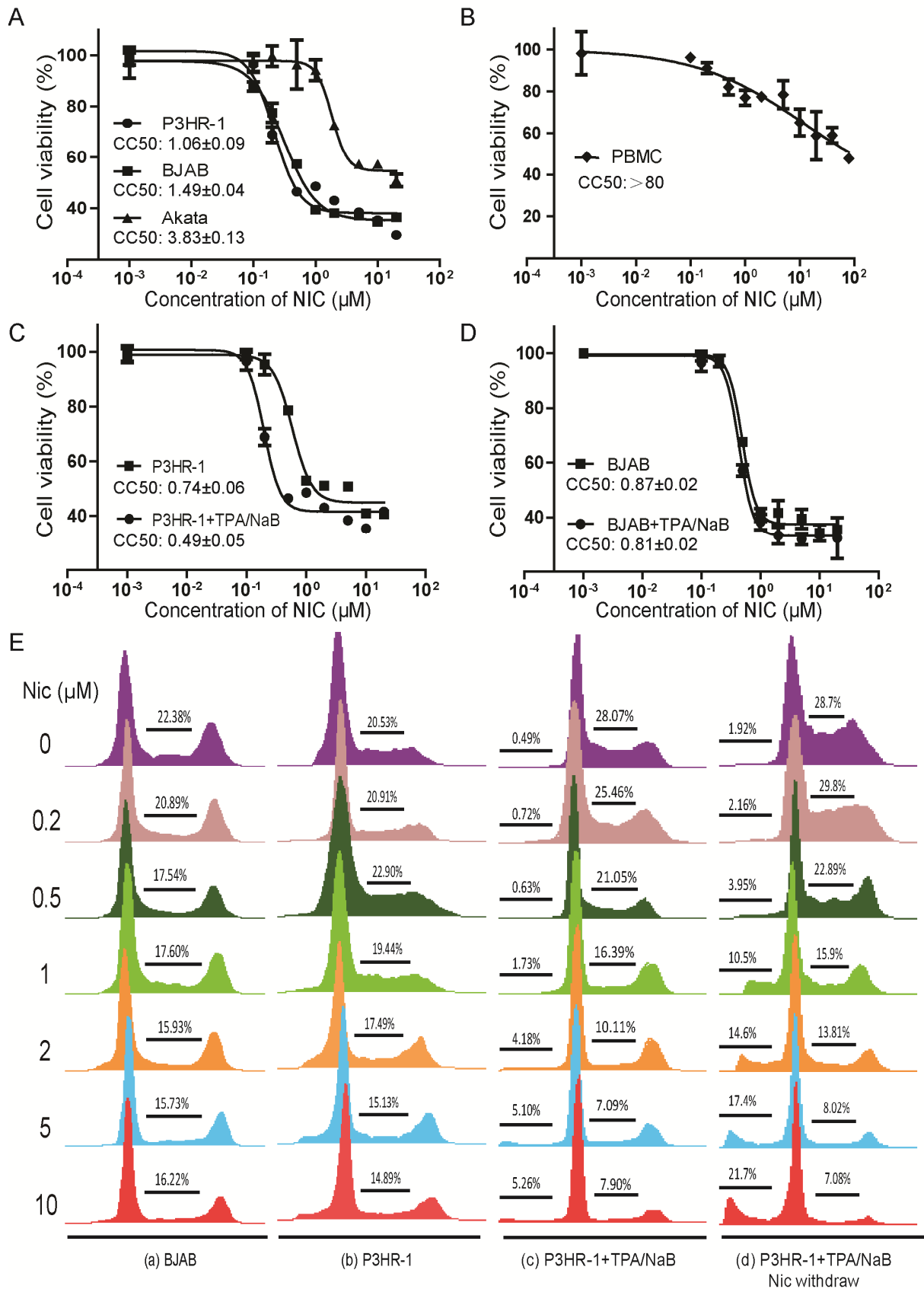
662 experiments.
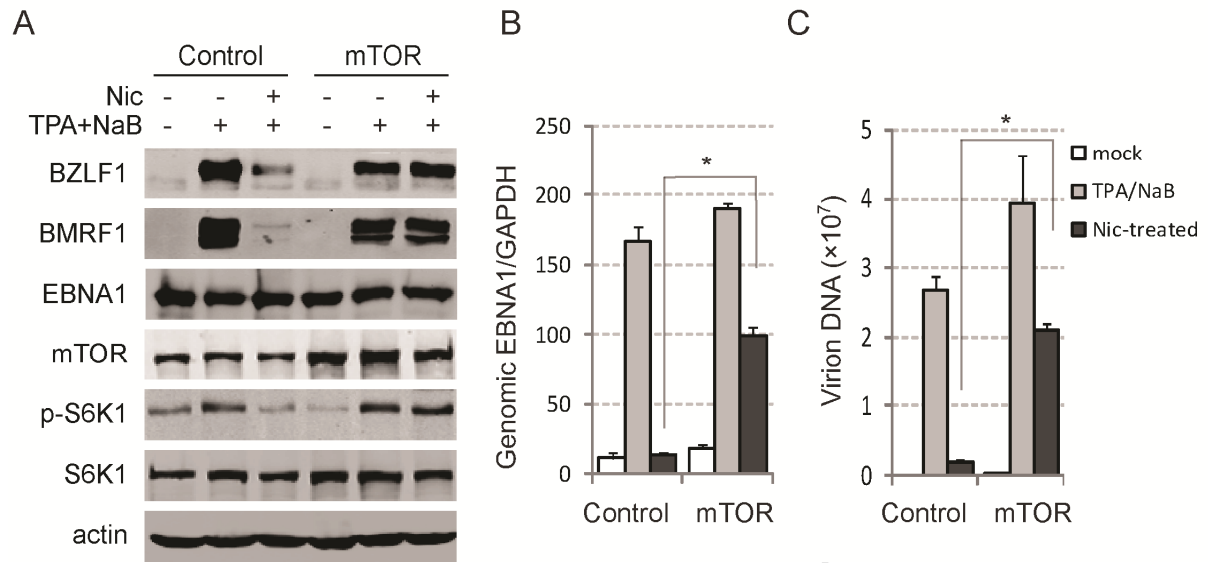
663

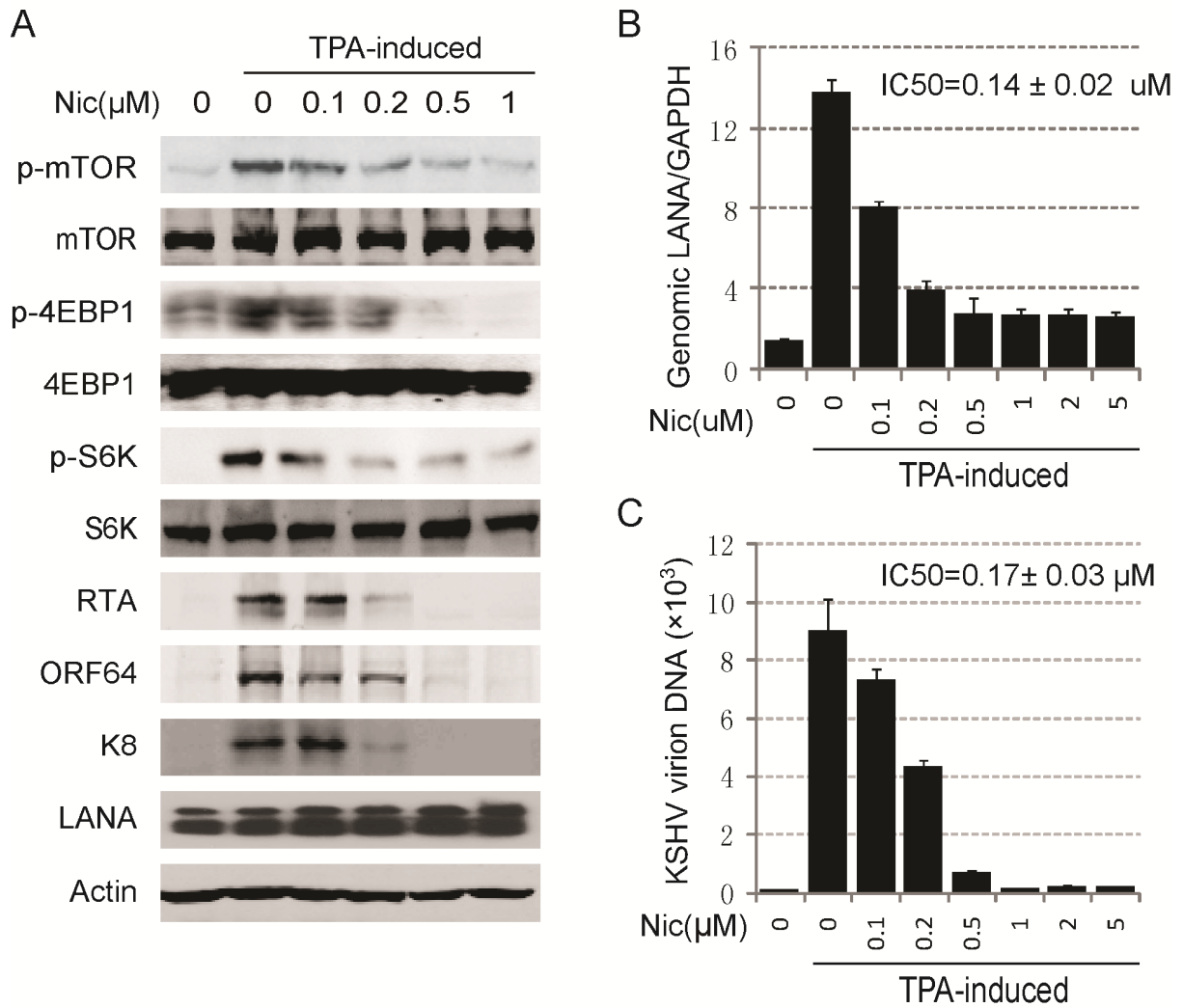Hu et al. Figure 1

Hu et al. Figure 2

Hu et al. Figure 3

Hu et al. Figure 4

Hu et al. Figure 5

Hu et al. Figure 6

A

TPA-induced

Nic(μM) 0   0   0.1 0.2 0.5   1

p-mTOR
mTOR
p-4EBP1
4EBP1
p-S6K
S6K
RTA
ORF64
K8
LANA
Actin

B

IC50=0.14 ± 0.02 uM

Genomic LANA/GAPDH

Nic(uM) 0   0 0.1 0.2 0.5 1 2 5

TPA-induced

C

IC50=0.17± 0.03 μM

KSHV virion DNA (×10³)

Nic(μM) 0   0 0.1 0.2 0.5 1 2 5

TPA-induced

Highlights

Niclosamide suppresses EBV lytic replication through disrupting mTOR activation.

Niclosamide induces irreversible cell cycle arrest in lytically EBV-infected cells.

Niclosamide is a promising therapy against infectious diseases of γ-herpesviruses.