BRIEF REPORT

Evaluation of kinase inhibitors as potential therapeutics for flavivirus infections

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Abstract

The recent introduction of Zika virus (ZIKV), the recurrence of dengue virus (DENV), and the lethality of yellow fever virus (YFV) have had a significant impact on Brazilian society and public health. Here, we targeted two cellular kinases implicated in cell proliferation and cancer that are also important for viral replication: mitogen-activated protein kinase kinase (MEK) and Src. We used two MEK inhibitors – trametinib and selumetinib – and two Src inhibitors – saracatinib and bosutinib – to inhibit ZIKV, DENV, and YFV replication in cell culture. The cytotoxicity of the four inhibitors was determined by the observation of abnormal morphology and quantification of adherent cells by crystal violet staining. The antiviral activity of these drugs was assessed based on the reduction of plaque-forming units in cell culture as evidence of the inhibition of the replication of the selected flaviviruses. All four inhibitors showed antiviral activity, but among them, trametinib was the safest and most efficacious against all of the viruses, inhibiting the replication of ZIKV and YFV by 1000-fold, and DENV2/3 by nearly 100-fold. This pan-antiviral effect shows that trametinib could be repurposed for the treatment of flaviviral infections.

From 2014 to 2018, the incidence of flavivirus infections increased in Brazil. Suspected cases of dengue fever were estimated at >4.2 million, Zika fever cases were estimated at >241,000, and yellow fever cases were estimated at >10,000 (Supplementary Table 1, Supplementary Fig. 1) [1–9]. Zika virus (ZIKV) infection has been associated with congenital defects in fetuses and newborn children [10], and no specific

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treatment has been developed to date. Antiviral compounds that target cellular pathways are less likely to select resistant strains than those targeting the virus, and they can potentially affect all viruses that use the same pathway. Mitogen-activated protein kinase kinase (MEK) and Src are cellular kinases that participate in cell proliferation, development, differentiation, and survival [11], and they are also important for the replication of several viruses [12, 13]. Using high-throughput screening assays, the MEK1/2 inhibitor U0126 [14] and the Src inhibitors dasatinib and saracatinib [15] have been identified as potential anti-dengue compounds. They were originally designed for the treatment of cancer but have since been shown to inhibit viruses of other families. Our team has already shown that phosphorylation of the extracellular signal-regulated kinase (ERK) by MEK is induced by dengue virus (DENV) and yellow fever virus (YFV) infection and that it is important for viral replication and assembly in cell culture and mouse models [16, 17]. Therefore, we decided to test the antiviral activity of MEK1/2 inhibitors that are currently undergoing clinical trials for cancer – selumetinib and trametinib – and the Src inhibitors saracatinib and bosutinib against Brazilian strains of ZIKV and DENV and the YFV vaccine strain.

First, drug toxicity in cell culture was evaluated based on cell viability using a crystal violet assay as described

previously [18]. Selumetinib, trametinib, saracatinib, and bosutinib (purity >99% for all) were purchased from Selleckchem (Houston, TX, USA), resuspended in dimethyl sulfoxide (DMSO) (Merck, USA), and stored at −20 °C. BHK-21 and Vero cells were cultured in 96-well plates with Dulbecco's modified Eagle's medium (DMEM; Cultilab, SP, Brazil), supplemented with 5% fetal bovine serum (FBS; Cultilab, SP, Brazil) and antibiotics, at 37 °C with 5% CO_2. They were treated once with increasing concentrations of the inhibitors or DMSO as a control, and the medium was maintained for 24 or 48 h for the antiviral assays described below. Then, the ZIKV Asian strain PE-243, which was isolated from a patient with mild symptoms in the city of Recife, Brazil, in 2015 [19], DENV2 PI59 [20], DENV3 MG20 [21], and the YFV 17DD vaccine strain [22] were propagated in C6/36 cells as described previously [17], and virus pools were stored at −80 °C. Infections were carried out in BHK-21 and Vero cells cultured in 96-well plates with DMEM supplemented with 5% FBS and antibiotics and incubated at 37 °C with 5% CO_2. The virus titer was determined by measuring plaque-forming units (PFU/ml) in Vero cells overlaid with 1.5% carboxymethylcellulose (CMC; Synth, SP, Brazil) in supplemented DMEM and incubated for 4–5 days. Cells were fixed with 3.7% formaldehyde, and viral plaques were visualized with 1% crystal violet solution. All experiments were repeated at least three times. The results were analyzed and graphics were generated using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA). Comparisons between means were analyzed using Student's t-test, considering $p < 0.05$ significant.

Since our previous findings have already shown the effect of selumetinib on DENV [16], we decided to test selumetinib on ZIKV using BHK-21 cells, which are permissive to many flaviviruses. BHK-21 cells were treated with an increasing twofold series of concentrations of 5–160 μM

selumetinib or saracatinib or 2.5–80 μM bosutinib for 24 h. Light microscopy examination showed no cytotoxic effect of selumetinib (40 μM), although a slight change in morphology was observed when cells were incubated with saracatinib (20 μM) and bosutinib (5 μM). Quantification of cell viability using a crystal violet assay and regression analysis to determine the 50% cytotoxic concentration (CC_{50}) showed that bosutinib had the highest toxicity (Fig. 1a, Table 1). CC_{50} and cell morphology were used to choose the drug concentrations for antiviral assays.

To assess antiviral activity, BHK-21 cells were infected with ZIKV PE-243 at an MOI of 0.1 and were incubated for 1 h to allow virus penetration. The cells were then washed and treated with inhibitors for 24 h, after which the supernatant was recovered to measure the virus titer by determining the number of plaque-forming units. Increasing concentrations of selumetinib (2.5–40 μM), saracatinib (1.2–20 μM), and bosutinib (0.3–5 μM) were tested.

All inhibitors reduced the viral titer in a dose-dependent manner. The reduction with selumetinib and saracatinib was more than 10-fold ($p < 0.001$), and with bosutinib it was more than 100-fold ($p < 0.001$) when the maximum concentrations were compared against controls treated with DMSO (Fig. 1b). Regression analysis to determine the 50% effective concentration (EC_{50}) showed that bosutinib and selumetinib had stronger antiviral activity than the other two compounds (Table 1). Because the selectivity index (SI), determined by calculating the CC_{50}/EC_{50} ratio for each inhibitor, showed that bosutinib had the lowest margin of safety, we decided to exclude it (Table 1).

Recent studies have more commonly used Vero cells than BHK-21 cells for ZIKV production, probably because they produce slightly higher and more stable viral titers (internal report). Therefore, we chose this cell line to test the effect of MEK1/2 inhibitors that are currently in clinical trials on
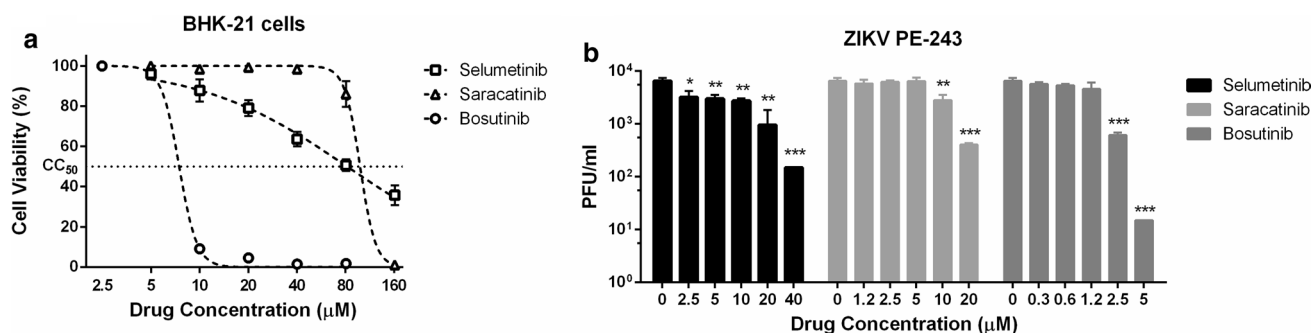
Fig. 1 Cytotoxicity in BHK-21 cells and antiviral activity of selumetinib, saracatinib, and bosutinib against ZIKV. a) BHK-21 cells were treated for 24 h with either DMSO or increasing concentrations of selumetinib, saracatinib, or bosutinib. Cell viability was quantified by crystal violet assay and represented as a percentage of the control and adjusted to a non-linear regression. CC_{50}: 50% cytotoxic concen-

tration. b) BHK-21 cells were infected with ZIKV PE-243 at an MOI of 0.1 and treated for 24 h with DMSO or increasing concentrations of selumetinib, saracatinib, or bosutinib. Viral titers were determined by plaque assay (PFU/ml). The results are the average of three experiments where *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ when compared to controls using a two-tailed Student's t-test.

Table 1 Cytotoxicity and antiviral activity of MEK and Src inhibitors

|	BHK-21 cells (μM)		
	Selumetinib	Saracatinib	Bosutinib
CC_{50} 24 hours (μM)	81.22	97.70	7.48
EC_{50} ZIKV PE-243 (μM)	5.19	9.48	1.48
SI (CC_{50}/EC_{50} ratio)	15.65	10.31	5.05
	Vero cells (μM)		
	Selumetinib	Trametinib	Saracatinib
CC_{50} 48 hours (μM)	177.50	173.70	16.53
EC_{50} ZIKV PE-243 (μM)	11.67	3.03	4.75
SI (CC_{50}/EC_{50} ratio)	15.21	57.33	3.48
EC_{50} DENV2 PI59 (μM)	7.82	2.46	-
SI (CC_{50}/EC_{50} ratio)	22.70	70.61	-
EC_{50} DENV3 MG20 (μM)	31.80	6.33	-
SI (CC_{50}/EC_{50} ratio)	5.58	27.44	-
EC_{50} YFV 17DD (μM)	27.91	7.91	-
SI (CC_{50}/EC_{50} ratio)	6.36	21.96	-

CC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective concentration; SI, selectivity index

ZIKV and other flaviviruses. Vero cells were treated with increasing twofold concentrations of 5–160 μM selumetinib or trametinib or 2.5–80 μM saracatinib for 24 or 48 h. Light microscopy examination of the cells treated for 24 or 48 h with selumetinib (40 μM) showed no changes. Saracatinib (20 μM) treatment resulted in slight changes in cell morphology, while trametinib (20 μM) treatment resulted in some punctual cell agglomeration. The CC_{50} values after 48 h of treatment indicated that saracatinib had the highest toxicity (Fig. 2a, Table 1). Vero cells were more tolerant to MEK inhibitors and more sensitive to the Src inhibitor when compared with BHK-21 cells. For antiviral assays, Vero cells were infected with ZIKV PE-243 at an MOI of 0.1 and incubated for 1 h to allow virus penetration. Then, the cells were washed and treated with inhibitors for 24 h with non-toxic concentrations (5–40 μM), after which the supernatant was recovered to measure the virus titer by determining the number of plaque-forming units. Treatment showed a 1000-fold reduction in viral titer with selumetinib (40 μM, $p < 0.001$) or trametinib (20 μM, $p < 0.001$), whereas saracatinib treatment (20 μM, $p < 0.001$) yielded only a 10-fold reduction (Fig. 2b).

Selumetinib (40 μM) and trametinib (20 μM) also reduced the viral titer of DENV2 PI59, DENV3 MG20, and the YFV 17DD vaccine strain ($p < 0.01$; Fig. 2c and d, Table 1). DENVs were tested at 48 h because of their longer replication cycle seen in viral replication curves (data not shown). Analysis of EC_{50} and SI values showed that trametinib had the highest antiviral efficacy and safety margins against the four flaviviruses (Table 1). Trametinib (20 μM) was also effective against the ZIKV African strain

MR766 (10-fold reduction, $p < 0.001$), although this strain originally obtained from a rhesus monkey was more adapted to culture and should be thoroughly evaluated. Finally, no nonspecific virucidal effect was observed when viruses were pre-incubated with each inhibitor (data not shown).

In conclusion, we have shown a pan-antiviral effect of two MEK inhibitors, selumetinib and trametinib, against ZIKV, DENV2, DENV3, and YFV. This supports the importance of the MEK/ERK pathway for flavivirus replication. This is also the first report of the treatment of ZIKV with MEK inhibitors *in vitro*. Trametinib had the most promising efficacy and safety margin and has already been reported to affect other viruses by impairing functions related to ERK phosphorylation. Specifically, trametinib decreases phospho-ERK2 incorporation into HIV virions, which impairs uncoating and infectivity [23] and reduces replication of different influenza subtypes by blocking ERK-dependent nuclear export of viral ribonucleoproteins [24]. U0126, another MEK inhibitor, has also been reported to affect the infectivity of the hepatitis C virus and DENV particles due to reduced phospholipase activity [25]. ZIKV was inhibited by PHA-690509, a cyclin-dependent kinase inhibitor, which blocks the cell in the G_0/G_1 phase [26]. Likewise, trametinib also blocks cells is this stage [27]. These cellular mechanisms affected by MEK inhibitors may be the link between the MEK/ERK pathway and the flaviviral replication cycle. Future studies to identify them should focus on the specific cellular partners involved. Also, a recent study has suggested that selumetinib may be used in the treatment of COVID-19, as well as other MEK inhibitors with the potential to control the pathogenic pathways associated to SARS-CoV-2
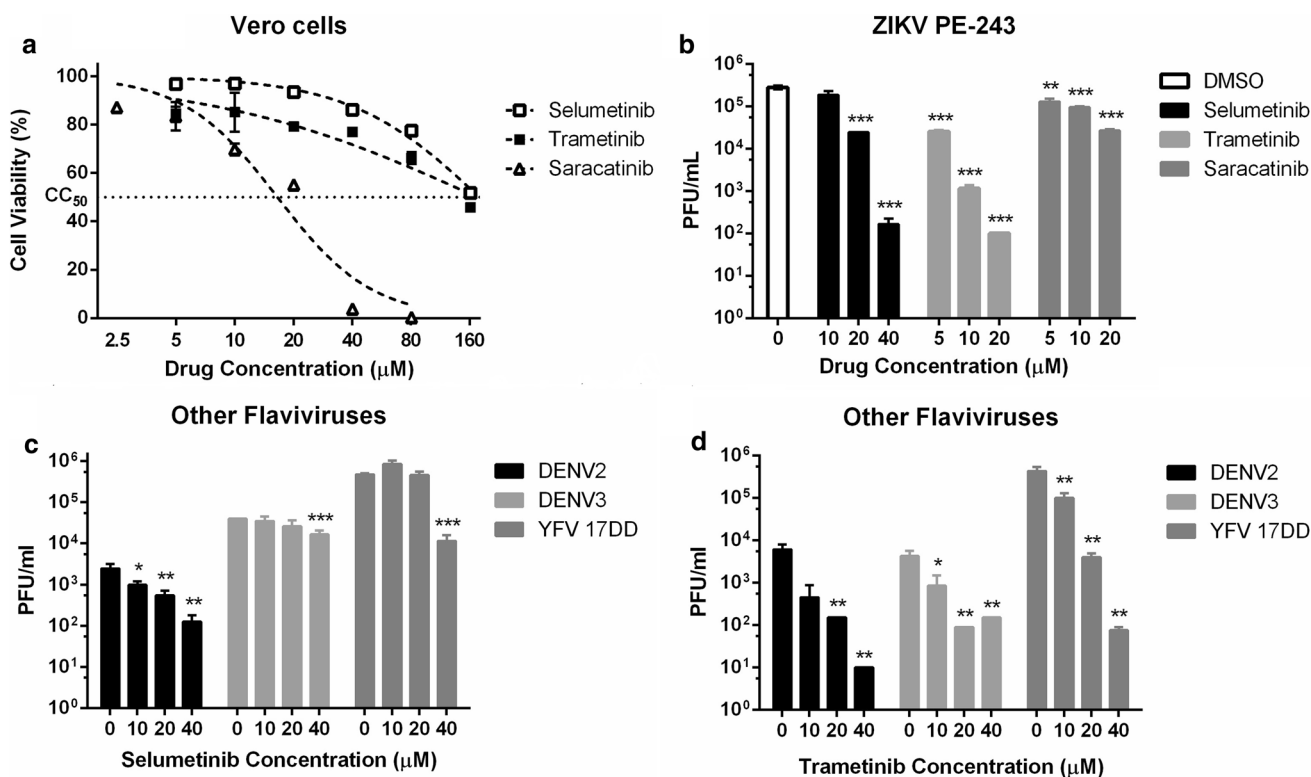
Fig. 2 Cytotoxicity in Vero cells and antiviral activity of selumetinib, trametinib, and saracatinib against ZIKV, DENV, and YFV. a) Vero cells were treated for 48 h with either DMSO or increasing concentrations of the indicated inhibitors. Cell viability was quantified by crystal violet assay and represented as a percentage of the control treated with DMSO and adjusted to a non-linear regression. CC_{50}: 50% cytotoxic concentration. b) Vero cells were infected with ZIKV PE-243 at an MOI of 0.1 and treated for 24 h with DMSO or increasing con-centrations of selumetinib, trametinib, or saracatinib. Viral titers were determined by plaque assay (PFU/ml). c–d) Vero cells were infected with DENV2, DENV3, or YFV at an MOI of 0.1 and treated with DMSO or increasing concentrations of selumetinib (c) or trametinib (d) for 24 h (YFV) or 48 h (DENVs). Viral titers were determined by plaque assay (PFU/ml). The results are the average of three experiments where $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ when compared to controls with a two-tailed Student's t-test.

[28]. Currently, there is no specific therapy or vaccine to treat ZIKV or DENV infections, and since trametinib has already been approved by the FDA (Mekinist) for melanoma treatment [29], it could be repurposed as a candidate for the treatment of flaviviral infection.

Availability of data and materials Viral samples will be available upon request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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