Antiviral Research 173 (2020) 104668

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

In silico drug repurposing for the identification of potential candidate molecules against arboviruses infection

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ARTICLE INFO

Keywords:
Dengue
Zika
Chikungunya
Virtual screening
Antiviral
Virus entry

ABSTRACT

Arboviral diseases caused by dengue (DENV), Zika (ZIKV) and chikungunya (CHIKV) viruses represent a major public health problem worldwide, especially in tropical areas where millions of infections occur every year. The aim of this research was to identify candidate molecules for the treatment of these diseases among the drugs currently available in the market, through *in silico* screening and subsequent *in vitro* evaluation with cell culture models of DENV and ZIKV infections. Numerous pharmaceutical compounds from antibiotics to chemotherapeutic agents presented high *in silico* binding affinity for the viral proteins, including ergotamine, antrafenine, natamycin, pranlukast, nilotinib, itraconazole, conivaptan and novobiocin. These five last compounds were tested *in vitro*, being pranlukast the one that exhibited the best antiviral activity. Further *in vitro* assays for this compound showed a significant inhibitory effect on DENV and ZIKV infection of human monocytic cells and human hepatocytes (Huh-7 cells) with potential abrogation of virus entry. Finally, intrinsic fluorescence analyses suggest that pranlukast may have some level of interaction with three viral proteins of DENV: envelope, capsid, and NS1. Due to its promising results, suitable accessibility in the market and reduced restrictions compared to other pharmaceuticals; the anti-asthmatic pranlukast is proposed as a drug candidate against DENV, ZIKV, and CHIKV, supporting further *in vitro* and *in vivo* assessment of the potential of this and other lead compounds that exhibited good affinity scores *in silico* as therapeutic agents or scaffolds for the development of new drugs against arboviral diseases.

1. Introduction

The incidence of dengue, Zika and chikungunya viral infections has grown dramatically around the world, becoming a global public health concern, with cases reported in more than 100, 80 and 40 countries, respectively (Calvo et al., 2015; Gardner et al., 2018; Mayer et al., 2017; Villegas et al., 2018; Walker et al., 2014; World Health Organization, 2018, 2017, 2016). Several vaccine candidates have been evaluated in preclinical and clinical trials (Hallengärd et al., 2014; López-Camacho et al., 2018; Roy et al., 2014; Smalley et al., 2016). However, there are no licensed vaccines against Zika and chikungunya and there exists uniquely one registered vaccine against dengue, called Dengvaxia, which only confer partial immunity and has some safety warnings (Aguiar and Stollenwerk, 2018; Flasche et al., 2016; Guzman et al., 2013; Halstead, 2017; Scherwitzl et al., 2017; Whitehead and Subbarao, 2017). The absence of approved antiviral drugs to combat these diseases (Low et al., 2017; Tomlinson et al., 2009) and the increase in the endemic zone of the vectors due to the climate change and unplanned urbanization (Ebi and Nealon, 2016; Liu-Helmersson et al., 2016), evidence the need for the development of potent anti-dengue drugs.

Flaviviruses such as dengue (DENV) and Zika (ZIKV) (belonging to the *Flaviviridae* family), and alphaviruses such as chikungunya (CHIKV) (belonging to the *Togaviridae* family) are small, enveloped, single-stranded, positive-sense RNA viruses, transmitted primarily by *Aedes spp.* mosquitoes (Lazear et al., 2016; Weaver, 2014). As part of their viral replication cycle, DENV, ZIKV and CHIKV enter host cells via viral glycoprotein receptor-mediated endocytosis to use the machinery of the

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https://doi.org/10.1016/j.antiviral.2019.104668
Received 22 November 2019; Accepted 27 November 2019
Available online 28 November 2019
0166-3542/

Abbreviation

CHIKV chikungunya virus
CHIKV E1 Envelope 1
CHIKV E2 Envelope 2
CHIKV nsP2 Non-structural polyprotein 2
DENV dengue virus
DENV-2 dengue virus type 2
DENV-3 dengue virus type 3
DENV C Capsid
DENV NS2B/NS3 Non-structural protein 2B/non-structural protein 3 complex
DENV NS5 DENV non-structural protein 5
DENV2 E DENV-2 virus envelope glycoprotein
DENV3 NS5 DENV-3 non-structural protein 5
FFA focus-forming assay
GlycAc glycine acid
NS3 non-structural protein-3
nsP2 nonstructural polyprotein 2
PDB Protein Data Bank
ZIKV Zika virus
ZIKV C Capsid protein from Zika Virus
ZIKV E Domain III of envelope protein
ZIKV MTase ZIKV MTase
ZIKV NS1 Zika virus non-structural protein 1
ZIKV NS2B/NS3 NS2B-NS3 protease
ZIKV NS3 NS3 protease
ZIKV NS5 NS5 RNA-dependent RNA polymerase (RdRP)

infected cell to synthesize viral proteins and replicate their genome (Dai et al., 2016; Harrison, 2015; Marsh and Pelchen-Matthews, 1993; Más and Melero, 2013). The viral RNA genome encodes a single polyprotein containing three structural proteins incorporated into the virions (capsid (C), pre-membrane/membrane (prM/M) and envelope (E)), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) that coordinates virus replication, assembly and modulation of host defense mechanisms. The molecular mechanism responsible for the development of disease (Eglen et al., 2000) is mediated by viral proteins, which constitute target molecules in drug development (Bekerman and Einav, 2015; Klumpp and Crépin, 2014; Liang et al., 2016). Therefore, the purpose of this research was to identify *in silico* candidate molecules among known pharmaceuticals with the potential to bind DENV, ZIKV and CHIKV proteins to help to prioritize compounds for *in vitro* an *in vivo* evaluation; with further *in vitro* antiviral activity assessment of five selected compounds. This drug repurposing (or repositioning) strategy was used to accelerate the discovery of safe and efficient treatments for patients (Abdulla et al., 2009; Bastos and Coelho, 2014; Langedijk, 2016; Ma et al., 2013; Mehndiratta et al., 2016; O'Connor and Roth, 2005; Pushpakom et al., 2018; J. Zhang et al., 2017).

2. Methods

2.1. Viral proteins and ligands structures

The crystallographic coordinates of DENV, ZIKV and CHIKV proteins, with suitable resolution for docking studies (on average ≤ 2.0 Å), were downloaded from Protein Data Bank (PDB) (http://www.rcsb.org/) in pdb format, and prepared as described in our previous article (Montes-Grajales et al., 2016).

Small-molecule drug structures were obtained from the database of approved pharmaceuticals on DrugBank (http://www.drugbank.ca) as sdf files and converted to mol2 format using Open Babel (O'Boyle et al., 2011). The molecular structures were prepared using AutoDockTools 1.5.6 (Morris et al., 2009) by adding Kollman charges and polar hydrogen atoms and were saved in pdbqt files.

2.2. Virtual screening protocol

The virtual screening protocol was the same used by Cabarcas-Montalvo et al. (2016), which has been validated with experimental assays for DENV proteins, as well as for other datasets in our previous studies (Cabarcas-Montalvo et al., 2016; Montes-Grajales et al., 2016, 2013; Montes-Grajales and Olivero-Verbel, 2013). Blind docking technique was employed with the purpose of finding promising lead compounds in existing drugs to target DENV, ZIKV and CHIKV proteins according to their AutoDock Vina affinity score (kcal/mol) (Trott and Olson, 2010). The settings used were: number of modes of 20, an energy range of 1.5, and exhaustiveness equal to 20. Simulations were performed in triplicate, keeping the best pose for each run. Finally, average affinities for the best poses were taken as the final value.

Additionally, a heatmap was created using R (R Core Team, 2016) with the affinity scores between pharmaceuticals and viral proteins, by using the "heatmap.2" function included in the gplot library (Warnes et al., 2009). The range color was defined implementing RColorBrewer, assigning red from −12.0 kcal/mol to −7.0 kcal/mol (strong and moderate), white from −7.0 kcal/mol to −6.0 kcal/mol (weak), and blue to affinities greater than −6.0 kcal/mol.

2.3. Evaluation of protein−ligand interactions

The best pose of the small-molecule drugs with the best affinity scores for the tested viral proteins were isolated in AutoDock Tools 1.5.6 (Morris et al., 2009) from the AutoDock Vina docking resultant files and merged with the optimized protein structures in pdb format using Pymol (Seeliger and de Groot, 2010). Contact residues of these merged structures were analyzed with LigandScout 3.0 (Wolber and Langer, 2005), setting the interaction cutoff threshold to 7 Å (Montes-Grajales and Olivero-Verbel, 2013).

2.4. Assessment of antiviral activity in DENV and ZIKV infection in vitro

To assess the potential inhibitory effect of pranlukast, nilotinib, conivaptan, itraconazole, and novobiocin (Suppl. Table 1) on the infectivity of enveloped viruses, we performed experimental infections *in vitro* using a human monocytic cell line expressing the attachment factor DC-SIGN (U937-DC-SIGN), which is highly susceptible to DENV

Table 1
List of studied proteins.

Name	Abbreviation	PDB ID	Organism
DENV-2 virus envelope glycoprotein	DENV2 E	1OK8	DENV
DENV Capsid	DENV C	1R6R	DENV
DENV non-structural protein 2B/non-structural protein 3 complex	DENV NS2B/NS3	2FOM	DENV
DENV non-structural protein 5	DENV NS5	2J7U	DENV
DENV-3 non-structural protein 5	DENV3 NS5	5JJS	DENV
ZIKV Domain III of envelope protein	ZIKV E	5OMZ	ZIKV
ZIKV Capsid Protein from Zika Virus	ZIKV C	5YGH	ZIKV
ZIKV NS5 MTase	ZIKV MTase	5WXB	ZIKV
Zika virus non-structural protein 1	ZIKV NS1	5K6K	ZIKV
ZIKV NS2B-NS3 protease	ZIKV NS2B/NS3	5H4I	ZIKV
ZIKV NS3 protease	ZIKV NS3	5YOD	ZIKV
ZIKV NS5 RNA-dependent RNA polymerase (RdRP)	ZIKV NS5	5WZ3	ZIKV
CHIKV envelope 1	CHIKV E1	3N40	CHIKV
CHIKV envelope 2	CHIKV E2	3N44	CHIKV
CHIKV non-structural polyprotein 2	CHIKV nsP2	3TRK	CHIKV

Table 2
Small-molecule drugs with the best docking affinity scores (kcal/mol) for the studied DENV, ZIKV and CHIKV proteins.

Name	CID	Description	Docking affinity (kcal/mol)
DENV2 E			
Ergotamine	8223	Vasoconstrictor	−9.8 ± 0.2
Dihydroergotamine	10531	Analgesic/ vasoconstrictor	−9.4 ± 1.2
Nilotinib	644241	Antineoplastic	−9.3 ± 0.1
Antrafenine	68723	Analgesic/ antiinflammatory	−9.3 ± 0.5
Gliquidone	91610	Hypoglycemic	−9.1 ± 0.2
DENV C			
Pranlukast	4887	Antiasthmatic	−9.1 ± 0.1
Cabozantinib	25102847	Antineoplastic	−8.9 ± 0.0
Ciclesonide	6918155	Antiallergic/ antiinflammatory	−8.9 ± 0.0
Dihydroergotamine	10531	Analgesic/ vasoconstrictor	−8.8 ± 0.0
Nilotinib	644241	Antineoplastic	−8.8 ± 0.0
Zafirlukast	5717	Antiasthmatic	−8.8 ± 0.1
Nandrolone phenpropionate	229455	Anabolic	−8.8 ± 0.2
DENV NS2B/NS3 complex			
Nilotinib	644241	Antineoplastic	−10.6 ± 0.2
Imatinib	5291	Antineoplastic	−10.2 ± 0.1
Regorafenib	11167602	Antineoplastic	−10.1 ± 0.2
Ponatinib	24826799	Antineoplastic	−10.1 ± 0.2
Eltrombopag	66583167	Antithrombocytopenic	−9.5 ± 0.0
Sorafenib	216239	Antineoplastic	−9.5 ± 0.0
DENV NS5			
Conivaptan	151171	Vasopressin antagonist	−10.6 ± 0.0
Dutasteride	6918296	Prostatic hypertrophy agent	−10.3 ± 0.0
Natamycin	5284447	Antifungal	−9.9 ± 0.0
Tubocurarine	6000	Neuromuscular blocker	−9.9 ± 0.0
Ergotamine	8223	Vasoconstrictor	−9.7 ± 0.2
DENV3 NS5			
Conivaptan	151171	Vasopressin antagonist	−11.4 ± 0.0
Ergotamine	8223	Vasoconstrictor	−11.3 ± 0.0
Dihydroergotamine	10531	Analgesic/ vasoconstrictor	−11.0 ± 0.0
Diosmin	5281613	Vasoconstrictor	−11.0 ± 0.0
Irinotecan	60838	Antineoplastic	−11.0 ± 0.2
ZIKV E			
Ergotamine	8223	Vasoconstrictor	−9.3 ± 0.8
Dihydroergotamine	10531	Analgesic/ vasoconstrictor	−9.1 ± 0.0
Nilotinib	644241	Antineoplastic	−9.1 ± 0.1
Lomitapide	9853053	Antilipemic	−9.0 ± 0.1
Eltrombopag	66583167	Antithrombocytopenic	−8.9 ± 0.3
Conivaptan	151171	Vasopressin antagonist	−8.9 ± 0.1
Gliquidone	91610	Hypoglycemic	−8.9 ± 0.3
ZIKV C			
Nilotinib	644241	Antineoplastic	−8.9 ± 0.2
Dihydroergotamine	10531	Analgesic/ vasoconstrictor	−8.9 ± 0.1
Conivaptan	151171	Vasopressin antagonist	−8.2 ± 0.0
Setiptiline	5205	Antidepressant	−8.2 ± 0.0
Ergotamine	8223	Vasoconstrictor	−8.2 ± 0.4
ZIKV MTase			
Nilotinib	644241	Antineoplastic	−10.8 ± 0.6
Tolvaptan	443894	Vasopressin antagonist	−10.7 ± 0.0
Daunorubicin	30323	Antineoplastic	−10.7 ± 0.0
Doxorubicin	31703	Antibiotic	−10.7 ± 0.0
Viomycin	3037981	Antibiotic	−10.5 ± 0.2
ZIKV NS1			
Nilotinib	644241	Antineoplastic	−9.7 ± 0.4
Eltrombopag	66583167	Antithrombocytopenic	−9.6 ± 0.2
Telmisartan	65999	Antihypertensive	−9.4 ± 0.4
Adapalene	60164	Dermatologic	−9.3 ± 0.6
Dihydroergotamine	10531	Analgesic/ vasoconstrictor	−9.0 ± 0.1
ZIKV NS2B/NS3			
Teniposide	452548	Antineoplastic	−9.8 ± 0.1
Eltrombopag	66583167	Antithrombocytopenic	−9.4 ± 0.3
Nilotinib	644241	Antineoplastic	−9.4 ± 0.3
Etoposide	36462	Antineoplastic	−9.2 ± 0.1
Ergotamine	8223	Vasoconstrictor	−9.1 ± 0.3
Dihydroergotamine	10531	Analgesic/ vasoconstrictor	−9.1 ± 0.2
Ponatinib	24826799	Antineoplastic	−9.1 ± 0.1
ZIKV NS3			
Dihydroergotamine	10531	Analgesic/ vasoconstrictor	−10.2 ± 0.4
Zafirlukast	5717	Antiasthmatic	−9.8 ± 0.3
Conivaptan	151171	Vasopressin antagonist	−9.8 ± 0.0
Nilotinib	644241	Antineoplastic	−9.6 ± 0.2
Ergotamine	8223	Vasoconstrictor	−9.6 ± 0.2
ZIKV NS5			
Ergotamine	8223	Vasoconstrictor	−10.4 ± 0.0
Dihydroergotamine	10531	Analgesic/ vasoconstrictor	−10.4 ± 0.0
Dutasteride	6918296	5α-reductase inhibitor	−10.0 ± 0.4
Nilotinib	644241	Antineoplastic	−10.0 ± 0.1
Ergoloid mesylate	87068835	Vasodilator	−9.9 ± 0.1
Conivaptan	151171	Vasopressin antagonist	−9.9 ± 0.1
CHIKV E1			
Natamycin	5284447	Antifungal	−10.7 ± 0.0
Tubocurarine	6000	Neuromuscular blocker	−10.6 ± 0.0
Eplerenone	443872	Diuretic	−10.6 ± 0.0
Nilotinib	644241	Antineoplastic	−10.6 ± 0.1
Ergotamine	8223	Vasoconstrictor	−10.5 ± 0.0
Dihydroergotamine	10531	Analgesic/ vasoconstrictor	−10.5 ± 0.0
CHIKV E2			
Nilotinib	644241	Antineoplastic	−10.9 ± 0.1
Conivaptan	151171	Vasopressin antagonist	−10.8 ± 0.2
Tubocurarine	6000	Neuromuscular blocker	−10.6 ± 0.5
Ergotamine	8223	Vasoconstrictor	−10.6 ± 0.0
Etoposide	36462	Antineoplastic	−10.6 ± 0.0
Eltrombopag	66583167	Antithrombocytopenic	−10.6 ± 0.1
Tigecycline	54686904	Antibiotic	−10.6 ± 0.1
CHIKV nsP2			
Irinotecan	60838	Antineoplastic	−9.6 ± 0.1
Nilotinib	644241	Antineoplastic	−9.6 ± 0.1
Conivaptan	151171	Vasopressin antagonist	−9.5 ± 0.1
Dihydroergotamine	10531	Antimigraine	−9.4 ± 0.0
Zafirlukast	5717	Antiasthmatic	−9.3 ± 0.2
Telmisartan	65999	Antihypertensive	−9.3 ± 0.1

and ZIKV infection (Kraus et al., 2007; Montoya et al., 2018). All drugs were tested under conditions and concentrations that did not affect cell viability (Suppl. Fig. 1). A detailed description is provided in Suppl. Methods.

2.5. Fluorescence microscopy

The antiviral effect of pranlukast was additionally examined using the human hepatocyte cell line (Huh-7), widely described to be highly susceptible to DENV and ZIKV infection *in vitro* (Chan et al., 2016; Soto-Acosta et al., 2013). Additional characterization of the imaging method and infected cell count is given in Suppl. Methods.

2.6. Intrinsic fluorescence assay

To evaluate the potential interactions between pranlukast and nilotinib with some of the viral proteins identified after *in silico* analyses, we measured the intrinsic fluorescence intensity values (Byrd et al., 2013) of three recombinant proteins of DENV: envelope (recE), capsid (C), and the non-structural protein-1 (NS1). All three recombinant proteins used in this study: NS1 (The Native Antigen Company, Oxford), C (Sino Biological, Cat# 40262-V07E) and recE (in-house produced) were certified to be > 95% pure by manufacturers and in-house

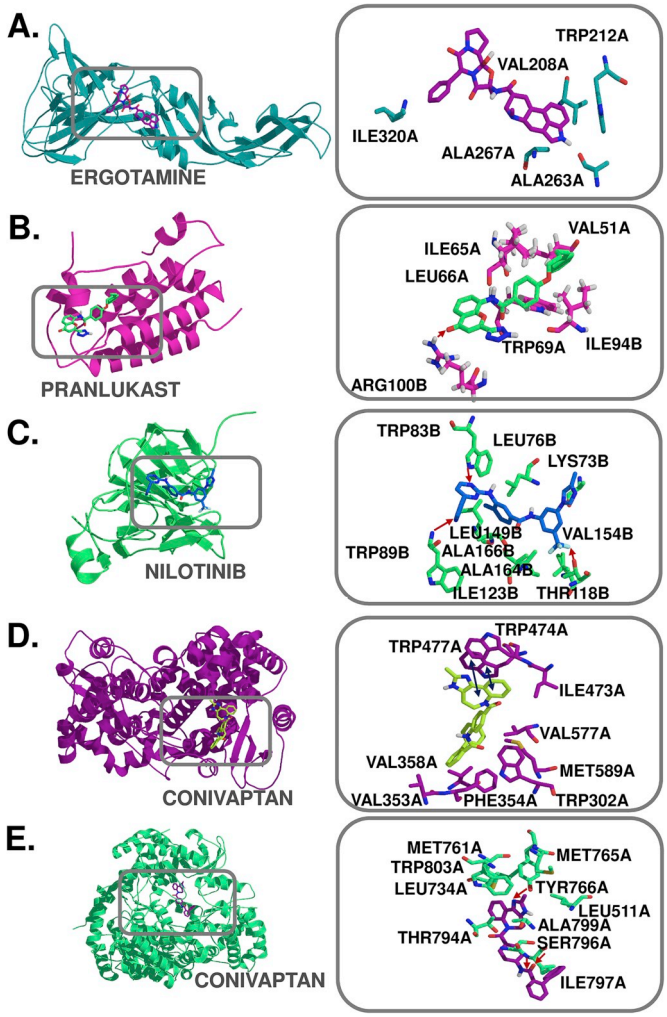

Fig. 1. Three-dimensional view of the overall structures (left) and predicted binding sites (right) of the protein-ligand complexes. A. DENV2 E-Ergotamine, B. DENV C-Pranlukast, C. DENV NS2B/NS3 protease-Nilotinib, D. DENV NS5-Conivaptan and E. DENV3 NS5-Conivaptan complexes. Red arrows represent hydrogen-bond acceptor features and blue arrows indicate aromatic ring interactions.

approaches as previously described (Beatty et al., 2015; Puerta-Guardo et al., 2019a) In addition, the presence and specificity of the recombinant protein was demonstrated by Western blot using specific α-NS1 (pan-flavivirus 2B7 mAb), α-capsid, α-envelope (pan-flavivirus 4G2 mAb) (Suppl. Fig. 2). Further information is presented in Suppl. Methods.

2.7. Statistical analyses

All statistical analyses and graphs were performed and generated using GraphPad Prism 6 software. Multiple comparison analyses between three or more groups were conducted using an ordinary two-way analysis of variance (ANOVA). Comparison between two groups were carried out using t tests (non-parametric) (Mann-Whitney test). Differences were considered significant for p values < 0.05.

3. Results

3.1. Viral proteins and ligand structures

Fifteen DENV, ZIKV and CHIKV proteins (Table 1) were found to have an available crystal structure with suitable resolution and were

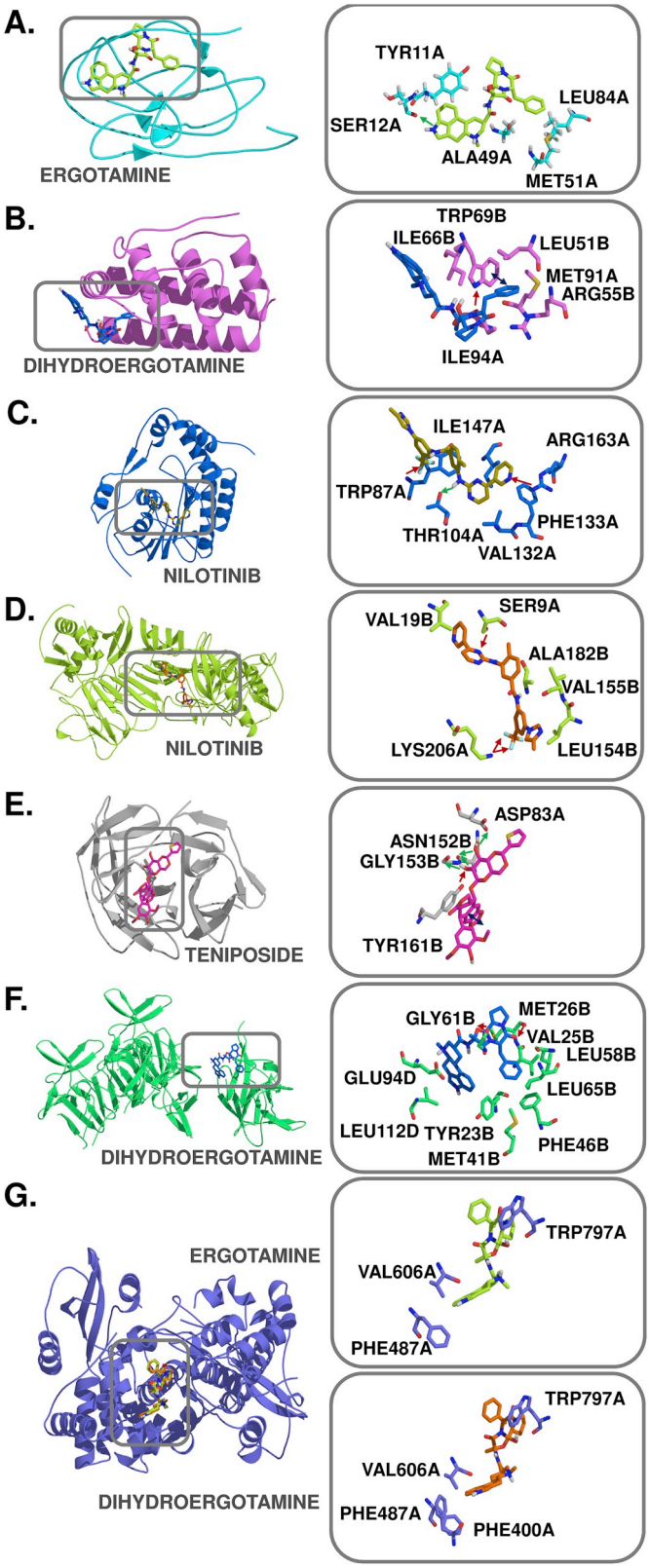

Fig. 2. Three-dimensional view of the overall structures (left) and predicted binding sites (right) of the protein-ligand complexes. A. ZIKV E-Ergotamine, B. ZIKV C-Dihydroergotamine, C. ZIKV NS5 MTase-Nilotinib, D. ZIKV NS1-Nilotinib, E. ZIKV NS2B/NS3-Teniposide, F. ZIKV NS3-Dihydroergotamine, G. ZIKV NS5-Dihydroergotamine (orange) and ZIKV NS5-Ergotamine (green). Red arrows represent hydrogen-bond acceptor features, blue arrows indicate aromatic ring interactions and green arrows represent hydrogen-bond acceptor and donor features.

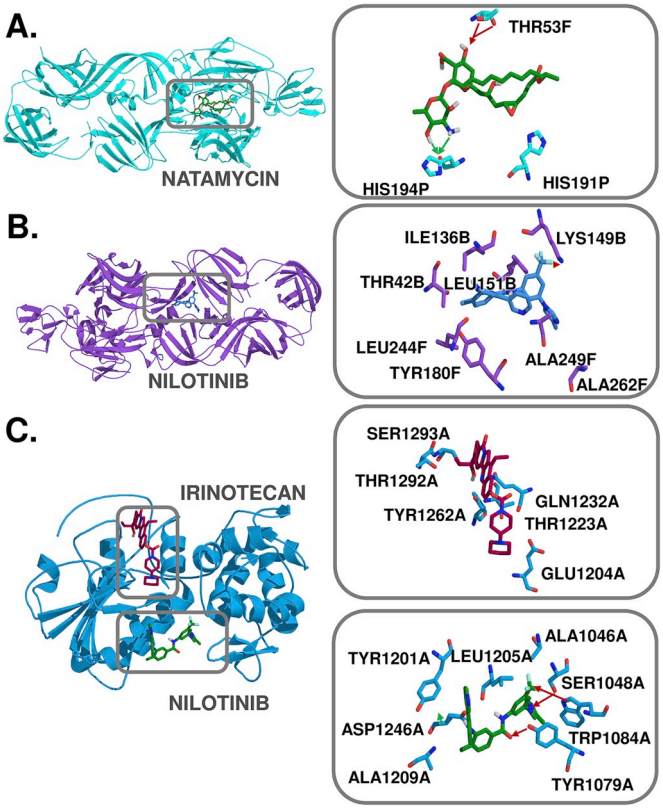

Fig. 3. Three-dimensional view of the overall structures (left) and predicted binding sites (right) of the protein-ligand complexes. A. CHIKV E1-Natamycin, B. CHIKV E2-Nilotinib, C. CHIKV nsP2-Irinotecan (magenta) and CHIKV nsP2-Nilotinib (green) complexes. Red and green arrows represent hydrogen-bond acceptor and donor features, respectively.

downloaded from PDB (http://www.rcsb.org/) (Bernstein et al., 1977). A total of 1,597 pharmaceutical compounds (Suppl. Table 2-4) were obtained from DrugBank (Law et al., 2014) and prepared according to our protocol (Cabarcas-Montalvo et al., 2016; Montes-Grajales et al., 2016, 2013; Montes-Grajales and Olivero-Verbel, 2013).

3.2. Small molecule drugs have differential affinity to viral proteins of DENV, ZIKV and CHIKV

AutoDock Vina affinity scores for small-molecule drugs with DENV, ZIKV and CHIKV proteins are presented in Suppl. Tables 2-4, respectively; and a summary with the best docking results per protein target are shown in Table 2. These ranged from −11.4 to −1.0 kcal/mol. Interestingly, several different types of drugs, including vasoconstrictors, analgesics, antineoplasics, anti-inflammatories, antibiotics and antihistamines, among others, presented high affinity scores (≤ −9.0 kcal/mol) for the viral proteins tested. To better visualize these results, a clustered heatmap based on docking affinity scores of DENV, ZIKV and CHIKV proteins against pharmaceuticals is shown in Suppl. Fig. 3.

Regarding DENV, the proteins DENV2 E, DENV C and DENV NS2B/NS3 exhibited their highest docking affinity scores with the vasoconstrictor ergotamine (−9.8 kcal/mol), the antiasmathic pranlukast (−9.1 kcal/mol) and the antineoplastic agent nilotinib (−10.6 kcal/mol), respectively. In addition, two DENV proteins displayed the highest affinity with the vasopressin antagonist conivaptan, with affinity scores of −10.6 kcal/mol for DENV NS5 and -11.4 kcal/mol for DENV3 NS5. Three proteins studied from CHIKV demonstrated highest binding affinity value: E1 with the antifungal natamycin (−10.7 kcal/mol), and E2 and nsP2 with nilotinib (−10.9 kcal/mol and −9.6 kcal/mol, respectively). ZIKV proteins NS1 and NS5 MTase obtained the best affinity score with nilotinib (−9.7 and −10.8 kcal/mol, respectively); NS5, C, and NS3 with dihydroergotamine (−10.4, −8.9 and −10.2 kcal/mol, respectively); E and NS5 with ergotamine (−9.3 kcal/mol and −10.4 kcal/mol, respectively), and the NS2/NS3 complex with teniposide (−9.8 kcal/mol).

3.3. Small molecules drugs interact with distinct viral proteins via hydrophobic interactions

Results obtained from LigandScout (Wolber and Langer, 2005) showed that most of the protein-ligand interactions between DENV, ZIKV and CHIKV proteins with the candidate pharmaceuticals are hydrophobic, with the presence of some aromatic interactions and hydrogen bonds. The contact residues and type of interactions of the complexes with the best affinity scores are presented in Figs. 1–3, and those for pranlukast are available in Suppl. Fig. 4.

3.4. Pranlukast reduces DENV and ZIKV infection of human monocytic cells and human hepatocytes in vitro, potentially blocking virus entry

Here, human monocytic cells (U937-DC-SIGN) were infected using a pre-mixed preparation of purified DENV or ZIKV with several concentrations of pranlukast, nilotinib, conivaptan, itraconazole and novobiocin (Suppl. Table 1; Fig. 4). Twenty-four hours post-infection, pranlukast was the only tested compound that significantly inhibited the infection of U937-DC-SIGN cells, as measured by flow cytometry using NS3 as a marker for viral replication (Fig. 4A). Compared to DMSO-treated cells that were infected with DENV (21%) or ZIKV (17%), pranlukast showed an important reduction in expression of NS3 in cells infected with either DENV (IC_{50} = 11.85 μM) or ZIKV (IC_{50} = 22.28 μM) (Fig. 4A). Other compounds such as conivaptan and novobiocin (Fig. 4C, E) showed promising inhibitory effects for both DENV and ZIKV infections (Fig. 4C, E). In contrast, nilotinib and itraconazole showed no antiviral activity against either DENV or ZIKV infection of human monocytic cells (Fig. 4B, D). With these results, we additionally characterized the antiviral activity of pranlukast in DENV and ZIKV infection *in vitro* by determining the percentage of infected human monocytic cells and also the amount of virus production using the standard Vero focus forming assay (FFU/mL). Pranlukast reduced both the amount of DENV and ZIKV-infected cells after 24 h post-treatment and also reduced the infectivity of virus production from treated cells (Fig. 5A–C). A similar inhibitory effect was achieved by different concentrations of pranlukast (range: 50–12.5 μM) in human monocytic cells infected with DENV and ZIKV using different MOIs (0.1, 0.5, 1)(Fig. 5D) or human hepatocytes in which pranlukast (50–12.5 μM) but not nilotinib (25–12.5 μM) inhibited DENV and ZIKV infection after 24 h post-treatment (Fig. 6A–C). The differences in the concentrations tested for both compounds resulted from the analysis of the cell viability test (Suppl. Fig. 1), which revealed that nilotinib reduced the viability of human monocytic cells (> 20% cytotoxicity) at concentrations between 100 and 50 μM, while, pranlukast only affected cell viability at 100 μM. Further, pranlukast appeared to affect the early stages of DENV and ZIKV infection, as the percentage of infected cells was markedly reduced when virus and pranlukast were simultaneously added to infect U937 cells (Fig. 7A), compared to an alternative protocol when pranlukast was added 2 h after infection was initiated (Fig. 7B). Additionally, pranlukast (50–12.5 μM) but not nilotinib blocked more than 90% of DENV attachment to the surface of human monocytic cells (Fig. 7C). Attached virus was removed after treatment with glycine acid, a buffer that dissociate cell-surface associated virus on cell membranes (Fig. 7C). Together, these data show that pranlukast inhibited the infection of human monocytic cells with DENV or ZIKV under the conditions tested and affected DENV absorption on cell membranes.

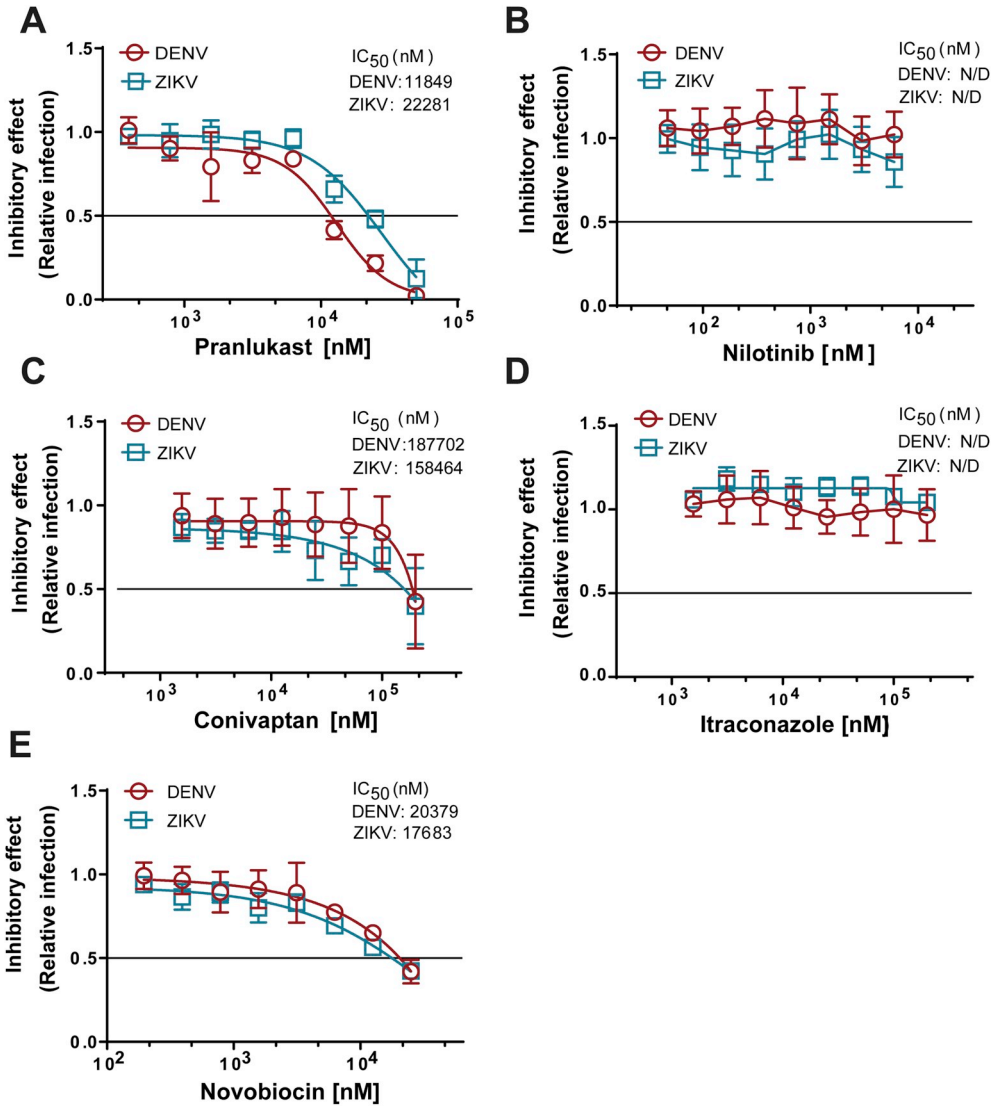

Fig. 4. Antiviral activity of *in silico* repurposed drug compounds against DENV and ZIKV infection of human monocytic cells *in vitro*. Human monocytic cells infected with DENV or ZIKV in the presence and absence of several concentrations (nM) of pranlukast, nilotinib, conivaptan, itraconazole and novobiocin (Table 1). Infection was determined after 24 h post-infection by detecting the viral replication marker NS3 using flow cytometry. Graphs represent the inhibitory effect for each compound against DENV and ZIKV infection of human monocytic cells. The inhibitory effect was expressed as the relative infection of non-infected cells (background) vs cells infected in the presence or absence of eight-different concentrations of each compound (nM). Analyses by nonlinear regression (curve fit) with variable slope predicted the IC_{50} as the measure of the antiviral effect potency of each compound against DENV or ZIKV infection of human monocytic cells. Each point represents the mean neutralization value from the two replicates, and the error bars depict the standard deviations. Neutralization experiments were repeated several times for pranlukast (n = 7), nilotinib (n = 13), conivaptan (n = 9), itraconazole (n = 4), and novobiocin (n = 4).

3.5. Pranlukast interacts with DENV proteins in vitro

Here, the intrinsic fluorescence values of three viral proteins E, C, and NS1 (200 ng/mL) were measured in the presence and absence of pranlukast (50 μM) and nilotinib (25 μM) (Fig. 8). All three recombinant proteins diluted in PBS(1x) and DMSO (0.5%) showed similar fluorescence values at the wavelengths included in the assay (300, 320, 340, 360 nm) (Fig. 8A–F). Interestingly, pranlukast significantly decreased the intrinsic fluorescence intensities of all three proteins, especially at the wavelength of 300 nm, where all three proteins showed a peak of maximum fluorescence, which were not saturating for the detection system (NS1: 25%; E: 30%; C: 20%; Fig. 8). Neither DMSO nor nilotinib affected the intrinsic protein fluorescence of any of these three proteins (< 1%)(Fig. 8A–F). Fluorescence values obtained with pranlukast and nilotinib alone are presented in Figure Suppl. Fig. 5. Together these results suggest that pranlukast interacts with these three viral proteins *in vitro*.

4. Discussion

Several already-approved drugs exhibited high *in silico* binding affinity for DENV, ZIKV and CHIKV proteins; including vasoconstrictors, analgesics, antineoplasics, anti-inflammatories, antibiotics and antihistamines. Interactions were predominantly hydrophobic with some hydrogen bonds, aromatic ring interactions and negative ionizable areas, which may contribute to stabilize the ligands at the target site and confer high affinity (Makhatadze and Privalov, 1995; Patil et al., 2010; Young et al., 2007).

Envelope proteins of DENV, ZIKV and CHIKV, involved in virus binding and membrane fusion, were found to have the best *in silico* affinity to many of the pharmaceutical compounds. Similar results were found for several non-structural proteins including NS2B, NS3, and NS5 from DENV and ZIKV; as well as for ZIKV NS1 and CHIKV nsP2.

Among the compounds tested *in vitro*, only the anti-asthmatic drug pranlukast exhibited a significant inhibition in the infection of human monocytic cells and human hepatocytes with DENV and ZIKV using *in vitro* cell culture models, potentially interfering with virus binding and/or entry into target cells via direct interaction with some viral proteins such as NS1, envelope and capsid. As a leukotriene receptor antagonist, pranlukast has been widely used to prevent seasonal virus-induced asthma exacerbation (Keam et al., 2003; Kim et al., 2016). However, the potential antiviral activity of pranlukast had not been addressed. These findings suggest that approved drugs available on the market may have potential therapeutic application against replication/transmission of arboviruses.

The *in silico* screening showed that pranlukast may interact with several viral targets (Suppl. Fig. 4). The interactions among those complexes appear to be characterized by hydrophobic interactions with

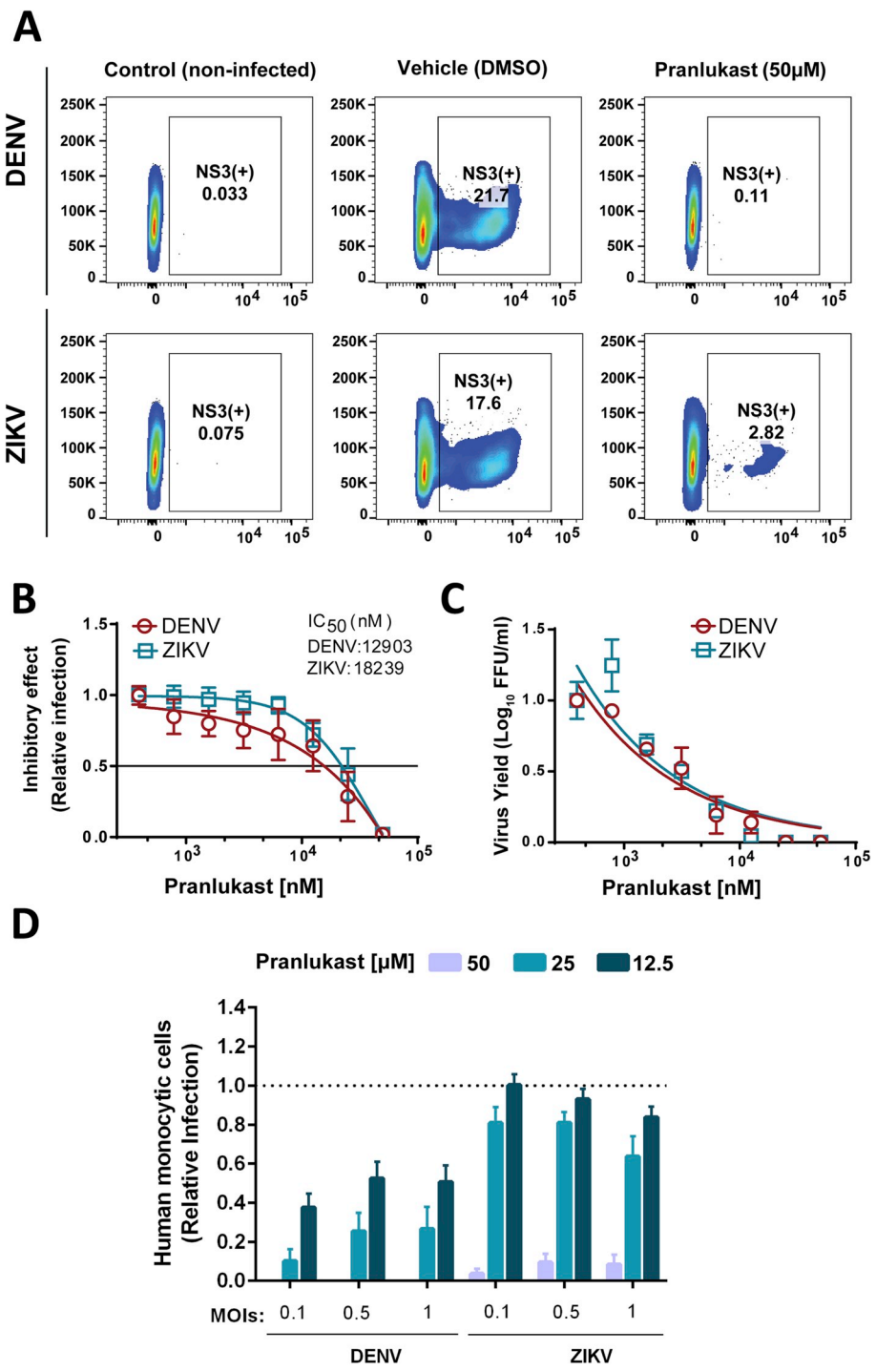

Fig. 5. Pranlukast reduces DENV and ZIKV infection of human monocytic cells and production of virus infectious particles. A. Representative cell counter plots for DENV and ZIKV infection of untreated, DMSO-treated and pranlukast-treated (50 μM) U937 DC-SIGN-expressing cells measured by flow cytometry using NS3 expression (gate NS3 +) as an active flavivirus replication marker. B. Inhibitory effect of pranlukast (50–0.390 μM) on DENV and ZIKV infection of U937-DC-SIGN cells at 24 h post-treatment. Non-infected cells and cells infected in the presence of DMSO (vehicle) were used as baseline for relative infection. Data represent mean ± standard error of the mean (SEM) of five individual experiments run in duplicate. C. Vero focus forming assay to detect virus infectious particles in supernatants collected from U937-DC-SING infected with DENV and ZIKV in the presence and absence of pranlukast (50–0.390 μM). Data represent mean ± SEM of two different experiments run in duplicate. D. Effect of pranlukast and nilotinib on DENV and ZIKV infection of human monocytic cells at different MOIs. U937-DC-SIGN cells were infected with a pre-mixed of DENV or ZIKV (MOIs: 0.1., 0.5., 1) plus pranlukast (50, 25, 12.5 μM). Infection was evaluated after 24 h post-treatment using flow cytometry assay. No differences were detected between non-infected and Mock-infected cells as well as non-treated cells and DMSO-treated (drug vehicle control) cells infected with either DENV or ZIKV. Data represent mean ± SEM of five different experiments run in duplicate.

some hydrogen bonds and aromatic-aromatic ring interactions. Pranlukast exhibited three polar interactions with CHIKV E1 and CHIKV E2 (ARG247F, TYR180F and LYS181F), in the same binding pocket reported for other promising molecules able to inhibit CHIKV replication (Mishra et al., 2016), and two polar interactions with DENV2 E (SER29A and SER363A) and ZIKV E (LYS100A and PHE18A). Analyses by intrinsic fluorescence assay, showed an apparent interaction between pranlukast and three viral proteins such as E, NS1 and C, suggesting a potential antiviral mechanism of pranlukast against DENV and ZIKV.

Here, our *in vitro* data showed that pranlukast possesses inhibitory activity against DENV and ZIKV infection of human monocytic cells and also human hepatocytes cells, two main cellular targets described for DENV and ZIKV (Chan et al., 2016; Soto-Acosta et al., 2013). Additional results suggested that pranlukast inhibited DENV and ZIKV infection of human monocytic cells at different MOIs. This inhibitory effect was mediated by blocking virus attachment, which may abrogate virus entry. Interestingly, pranlukast interacts with DENV proteins that play critical roles not only in virus entry but also virus replication such as envelope (E) and capsid (C). Protein E and C, two major structural proteins of flavivirus, play important roles in host cell viral interactions and virus morphogenesis (Samsa et al., 2009; Zhang et al., 2017). Our data suggests that pranlukast binding to E protein, may diminish virus attachment to the cell surface leading to reduced DENV and ZIKV infection in human cells. Surprisingly, pranlukast binds to NS1, a secreted viral protein important for immune evasion and pathogenesis (Beatty

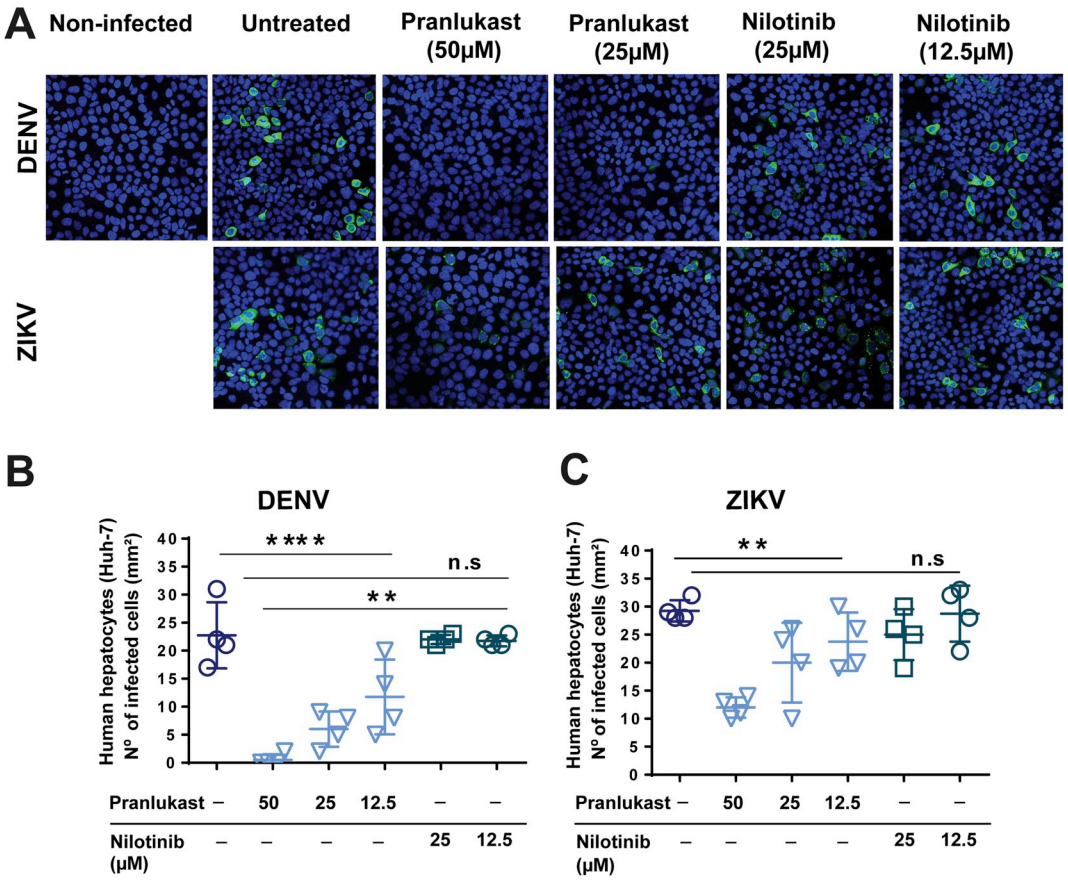

Fig. 6. Effect of pranlukast on DENV and ZIKV infection of human hepatocytes (Huh-7). (A) Representative images of Huh-7 cells infected with DENV (top panel) or ZIKV (lower panel) in the presence or absence of pranlukast and nilotinib after 24 h post-treatment. Nuclei stained with Hoechst (blue). Viral protein NS3 (green). B, C. Number of infected cells per standard area (μM^2) counted using ImageJ software analyses. (n = 4).

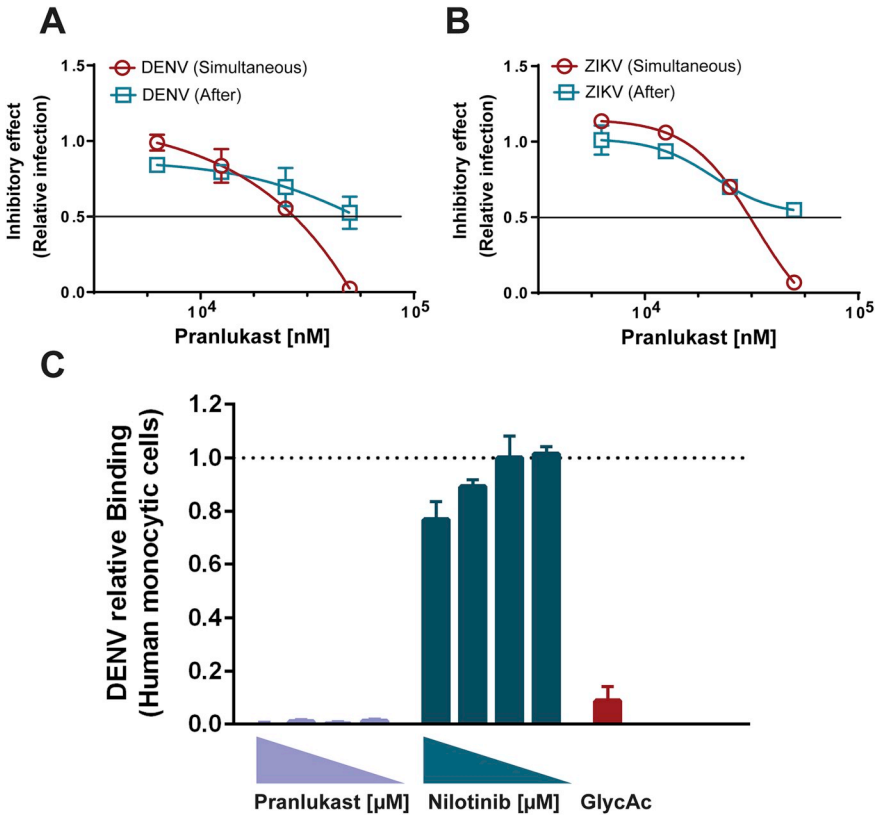

Fig. 7. Pranlukast reduces virus entry by blocking virus attachment to the surface of human monocytic cells. A-B. Pranlukast (50–6.25 μM) was added either simultaneously (pre-mixed virus plus drug) or after 2 h post-infection. DENV and ZIKV infection was examined after 24 h-post-treatment by flow cytometry analyses detecting the viral protein NS3. C. DENV attachment to cell surface was examined in the presence and absence of pranlukast (50–6.25 μM) and nilotinib (25–3.125 μM) with glycine acid (GlycAc) which removed any surface-bound, non-internalized virion from the outer cell membrane used as control.The amount of surface-bound virus was measured by flow cytometry using direct virus staining with an anti-envelope mAb (4G2-Alexa 568). DENV relative binding for all experimental conditions was estimated using the fluorescence signal obtained from human monocytic cells exposed to virus alone treated with DMSO and no virus treated cells used as background controls.

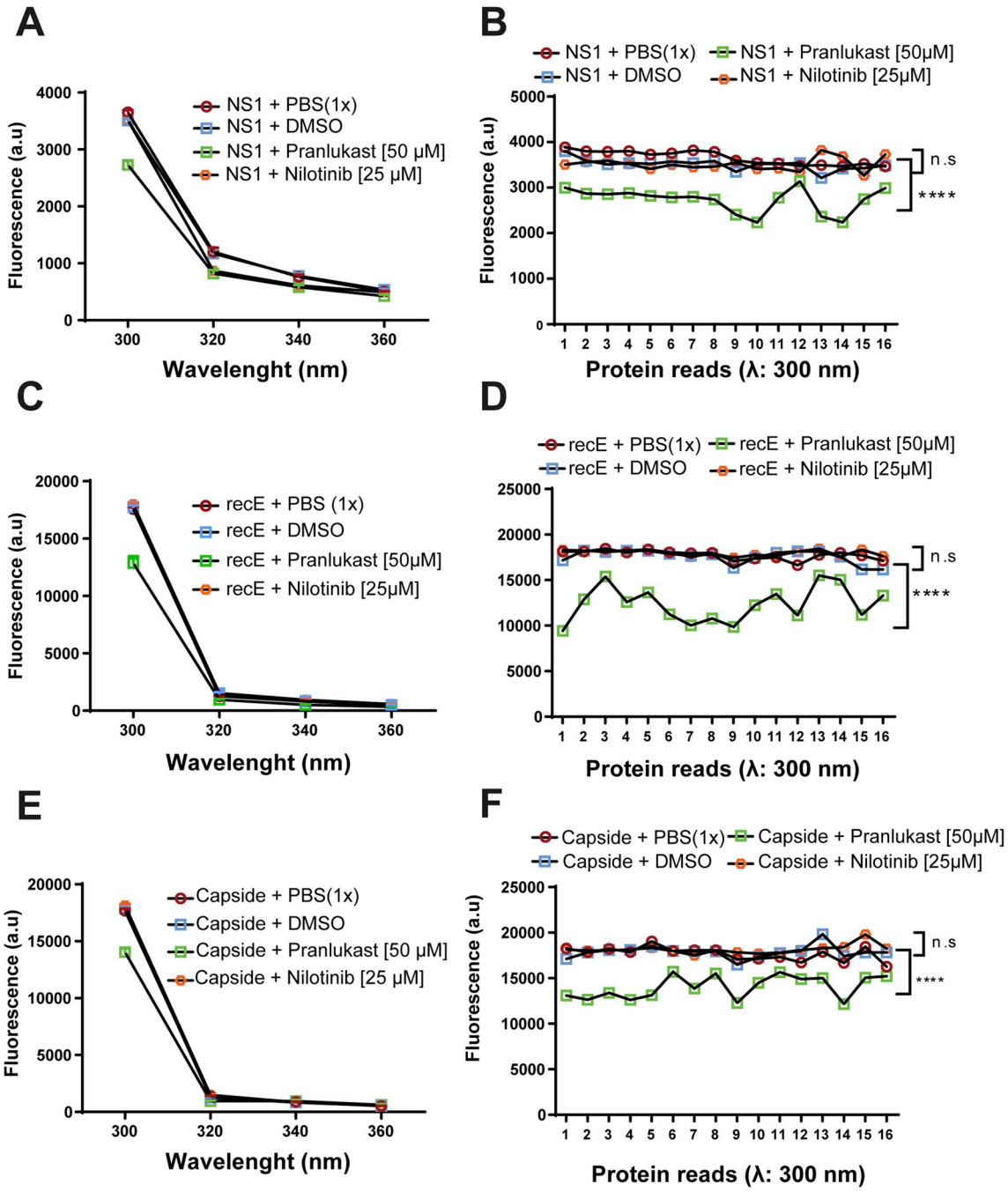

Fig. 8. Pranlukast interacts with DENV proteins. A, C, E. Intrinsic fluorescence values (expressed as fluorescence arbitrary units) of three purified DENV recombinant proteins: NS1, capsid and envelope (200 ng/mL) alone or in combination with DMSO (0.5%), pranlukast (50 μM) or nilotinib (25 μM) were scanned at 295 nm (excitation wavelength) and four different emission wavelengths (300, 320, 340, and 360 nm). B, D, F. Protein reads collected at one emission wavelength (300 nm). Data represent mean ± SEM of four different experiments including four replicates per run (total reads = 16). Statistical differences (Ordinary one-way ANOVA; Mann-Whitney non-parametric test) were considered significant as p values < 0.5.

et al., 2015; Glasner et al., 2018; Puerta-Guardo et al., 2019b). Further analyses using different molecular tools to evaluate its effect on virus internalization as well as viral RNA replication and also potential blocking effect on NS1 mediated-endothelial hyperpermeability would help to elucidate which steps of the viral replication cycle and viral pathogenesis are affected by pranlukast. Additionally, future studies to examine the antiviral effect of these compounds on the infection of other arboviruses such as chikungunya (CHIKV) should be addressed. Importantly, these results support our findings from the virtual screening that identified that pranlukast has a high affinity for the envelope proteins of DENV, ZIKV, and CHIKV.

A considerable number of antineoplastic agents, commonly used for cancer treatment, such as nilotinib (Schneider et al., 2015), displayed *in silico* interaction with DENV, ZIKV and CHIKV proteins (≤ -9.0 kcal/mol). The use of this kind of molecules as antivirals is not recommended due to their significant side effects (Afzal et al., 2015; Henβ et al., 2016; Stein et al., 1989). In this study, nilotinib showed no antiviral activity on DENV or ZIKV infection of human monocytes or hepatocytes *in vitro* neither bind to any of the viral proteins tested, however, it appeared to inhibit some virus attachment to the cell surface. Nilotinib has shown antiviral activity against cytomegalovirus infection and other related molecules such as sunitinib or erlotinib had inhibited dengue virus

which suggest that kinase inhibitors still represent interesting drugs with potential antiviral activity (Bekerman et al., 2017; Pu et al., 2018; Wolf et al., 2012).

In addition, other widely used drugs, such as the analgesic antrafenine (de Gara et al., 1982), the antifungals itraconazole, natamycin and ketoconazole (Carrillo-Muñoz et al., 1996; Zuckerman and Tunkel, 1994), the antibiotics novobiocin, cefoperazone, rolitetracycline, cefpiramide, piperacillin, dalfopristin and viomycin (Chant and Rybak, 1995; Finlay et al., 1951; Freel Meyers et al., 2003; Iakovlev and Kaplar-Vuchevats, 1994; Mazzola et al., 1980; Nolting et al., 1996; Pfaller et al., 2017), the antifungal natamycin (Balaguer et al., 2014), the antimycobacterial clofazimine (Cholo et al., 2017), the vasoconstrictor ergotamine (Tfelt-Hansen and Koehler, 2008), and the vasopressin antagonist conivaptan (Annane et al., 2009), among others, showed good affinity values with tested viral proteins. These proteins have shown to be critical for viral-cell membrane fusion (Allison et al., 2001; Chen et al., 1996; Crill and Roehrig, 2001; Garoff et al., 2004; Kielian et al., 2010; Modis et al., 2004; Voss et al., 2010; Weber et al., 2017), virus entry and recruiting of the viral genome during viral encapsulation and nucleocapsid creation (Ma et al., 2004; E. R. A. Oliveira et al., 2017; Rodenhuis-Zybert et al., 2010; Marcelo M Samsa et al., 2009), viral genome replication (Erbel et al., 2006; Lim et al., 2016; A. F. C. da S. Oliveira et al., 2017; Patil et al., 2010; Yap et al., 2007), and neutralization of cellular antiviral responses (Ahola and Merits, 2016; Akhrymuk et al., 2012; Fros et al., 2015, 2010). The analgesic antrafenine is also one of the most promising candidates due to its strong interaction with DENV2 E, DENV NS2B/NS3 and DENV3 NS5 proteins, along with all CHIKV and ZIKV studied proteins. Here, itraconazole did not inhibit the infection of DENV and ZIKV in human monocytic cells. Interestingly, conivaptan and novobiocin showed some antiviral activity against DENV and ZIKV at high concentrations. Novobiocin, a natural antibiotic that inhibits DNA gyrase, has been shown to poses some antiviral activity inhibiting vaccinia and herpesvirus (González-Molleda et al., 2012; Sekiguchi and Shuman, 1997). This finding points out these two compounds as potential candidates for future development of new antiviral molecules based on their chemical structures. Therefore, these drugs being less aggressive than anticancer agents could be considered for future *in vivo* tests aimed at evaluating their inhibitory activity against DENV, ZIKV, and CHIKV. This finding constitutes an initial approach for future studies to demonstrate the potential effect of these existing licensed drugs against human arboviral infections *in vitro* and *in vivo*.

In conclusion, these findings suggest that approved drugs available on the market may have potential therapeutic application against replication/transmission of arboviruses. Further, pranlukast showed promising antiviral activity against DENV and ZIKV primarily affecting the viral entry step when tested on human monocytic cells and human hepatocytes used as *in vitro* models for viral infection. Additionally, the antiviral activity of pranlukast against DENV and ZIKV may be rely on direct interaction with viral proteins critical for virus replication such as E, NS1, and C. Further analyses using more advanced tools (e.g. ultrafiltration, affinity chromatograph, biolayer interferometry, surface plasmon resonance) are needed to better characterize viral protein binding as well as binding affinity to FDA-approved drugs or new drug candidates. In a current scenario where no specific therapeutics and safe vaccines for these arboviral infections are available, the repurposing of approved drugs targeting viral proteins required for infection (Xie et al., 2015; Lim et al., 2016; de Silva et al., 2018; Glasner et al., 2018; Puerta-Guardo et al., 2019; Subudhi et al., 2018) still represents a promising approach for discovering new antiviral activities in FDA-approved drugs that can help to fight back important human diseases such as those caused by arboviruses.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Acknowledgements

The authors wish to thank the Administrative Department of Science, Technology and Innovation of Colombia [Grant: Colciencias No. 811-2018], Universidad Nacional Autónoma de México [Grant: Programa de Becas Posdoctorales en la UNAM 2016], Universidad Tecnológica de Bolívar [Grant: TRFCI-1P2016] and the National Institutes of Health [NIH grant R01 AI24493] for their financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104668.

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