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Short Communication

Host oxidative folding pathways offer novel anti-chikungunya virus drug targets with broad spectrum potential

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ABSTRACT

Alphaviruses require conserved cysteine residues for proper folding and assembly of the E1 and E2 envelope glycoproteins, and likely depend on host protein disulfide isomerase-family enzymes (PDI) to aid in facilitating disulfide bond formation and isomerization in these proteins. Here, we show that in human HEK293 cells, commercially available inhibitors of PDI or modulators thereof (thioredoxin reductase, TRX-R; endoplasmic reticulum oxidoreductin-1, ERO-1) inhibit the replication of CHIKV chikungunya virus (CHIKV) *in vitro* in a dose-dependent manner. Further, the TRX-R inhibitor auranofin inhibited Venezuelan equine encephalitis virus and the flavivirus Zika virus replication *in vitro*, while PDI inhibitor 16F16 reduced replication but demonstrated notable toxicity. 16F16 significantly altered the viral genome: plaque-forming unit (PFU) ratio of CHIKV *in vitro* without affecting relative intracellular viral RNA quantities and inhibited CHIKV E1-induced cell-cell fusion, suggesting that PDI inhibitors alter progeny virion infectivity through altered envelope function. Auranofin also increased the extracellular genome:PFU ratio but decreased the amount of intracellular CHIKV RNA, suggesting an alternative mechanism of action. Finally, auranofin reduced footpad swelling and viremia in the C57BL/6 murine model of CHIKV infection. Our results suggest that targeting oxidative folding pathways represents a potential new anti-alphavirus therapeutic strategy.

1. Short communication

Protein folding and structure-stabilizing, post-translational modifications, which are tightly constrained evolutionarily, represent processes for which RNA viruses must rely upon the host, thus offering an antiviral targeting strategy that leaves limited opportunity for escape by mutant viruses (Geller et al., 2007). There are numerous disulfide bond-forming cysteine residues throughout the alphavirus (family *Togaviridae*) envelope proteins, among which 16 in E1 (Mulvey and Brown, 1994), 12 in E2 (Snyder et al., 2012), and 2 in E3 (Parrott et al., 2009) are highly conserved across the genus (Voss et al., 2010). These disulfide bonds are important for both folding and native structure maintenance of arthritogenic alphavirus envelope proteins (Anthony et al., 1992; Carleton et al., 1997; Kaluza and Pauli, 1980; Mulvey and Brown, 1994; Phinney and Brown, 2000; Snyder et al., 2012). Disulfide bond formation and isomerization are mediated by a group of 20 redox enzymes containing a thioredoxin (TRX)-like domain, defined by a Cys-X-X-Cys motif in the active site, broadly called protein disulfide isomerases (PDIs) after their primary function in forming and isomerizing disulfide bonds (Ellgaard and Ruddock, 2005). Further, the oxidative state of PDI enzymes is modulated by various enzymes, prominently endoplasmic reticulum oxidoreductins (EROs) (Benham et al., 2013) and TRX-reductase (TRX-R) (Lundström and Holmgren, 1990).

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To investigate the potential for targeting oxidative folding pathways as a novel anti-alphavirus strategy, we investigated the effects of several compounds on chikungunya virus (CHIKV) replication *in vitro*: PDI-inhibitors PACMA31 (Bio-Techne, Pittsburgh, PA) (Xu et al., 2012) and 16F16 (Sigma-Aldrich, St. Louis, MO) (Hoffstrom et al., 2010), the FDA-approved TRX-R inhibitor auranofin (Bio-Techne, Pittsburgh, PA) (Furst, 1983), and ERO1 inhibitor EN460 (Darmstadt, Germany) (Blais et al., 2010). First, we established cytotoxicity curves in HEK293 cells (American Type Cell Culture Collection, Bethesda, MD) through 3 pooled independent (n = 3/treatment group per experiment) AlamarBlue cell viability assays (ThermoFisher Scientific, Waltham, MA) following manufacturer's instructions after 8, 12, 24, or 48 h of incubation with varying concentrations of compound. To establish dose-response curves, HEK293 cells were infected with a multiplicity of infection (MOI) of 0.01 plaque forming units (PFU)/cell of Caribbean CHIKV isolate YO123223 [World Reference Center for Emerging Viruses and Arboviruses (WRCEVA), University of Texas Medical Branch, UTMB]. One hour post-infection (HPI), cells were treated with various concentrations of PACMA31, 16F16, EN460, auranofin, or 1% DMSO. Supernatants were collected 8, 12, 24, and 48 HPI and viral titers were determined by plaque assay. Data were pooled from 2 independent experiments (n = 3/treatment group per experiment) for analysis. All treatments significantly decreased virus replication after high doses at 12, 24, and 48 HPI, but only EN460 produced a significant reduction at 8 HPI (Kruskall-Wallis, $p < 0.05$). EC_{log50} values increased over time, potentially due to compound degradation or depletion. Therapeutic indices (TIs) were calculated by dividing the cytotoxic-concentration-50% (CC_{50}) values by the compound concentration that reduced the log_{10} virus titer by 50% (EC_{log50}), both of which were determined by 5-factor sigmoidal-curve regression and point-of-inflection analyses based on cytotoxicity and dose-response curves, respectively (Table 1). Compounds were generally efficacious at concentrations higher than the CC_{50}, making it difficult to determine drug vs. toxicity effects, although log-scale inhibition of CHIKV replication was also observed at concentrations below the CC_{50}. The best performing compound was auranofin, which achieved the highest TI of 104.5 at 12 HPI. Curves fit to 24 h data are shown in Fig. 1 for comparisons of compound performance; all data available in Fig. S1.

Further, TIs were established for the compounds with the lowest EC_{log50} values, 16F16 and auranofin, against the emerging flavivirus, Zika virus (ZIKV strain FSS1302; WRCEVA, UTMB), and the related alphavirus Venezuelan equine encephalitis virus (VEEV strain ZPC738; rescued from an infectious cDNA clone) (Anishchenko et al., 2004) as described above using 1 and 2 independent experiments (n = 3/treatment group per experiment), respectively (Table 2). ZIKV replication was significantly decreased in a dose-dependent manner by both auranofin and 16F16 at 24 and 48 HPI (Kruskal-Wallis, $p < 0.05$), although point-of-inflection analyses for fitted curves were untenable (i.e. derivative incalculable) for 16F16 results at all time points and auranofin results at 8 and 12 HPI; treatment with auranofin resulted in a TI of 6.95 and 1.45 at 24 and 48 HPI, respectively. VEEV replication was also inhibited in a dose-dependent manner by both 16F16 and auranofin at all time points (Kruskal-Wallis, $p < 0.05$); 16F16 treatment produced TIs between 0.69 and 1.25, while auranofin treatment produced TIs between 1.45 and 16. While both compounds resulted in significant decreases in both VEEV and ZIKV titers, only auranofin showed specific inhibition of viral replication below the CC_{50}. Curves fit to 24 h data are shown in Fig. 2 to provide a representative visual comparison of compound performance.

We hypothesize that inhibiting oxidative folding pathways deregulates disulfide bond formation and isomerization within the alphavirus envelope proteins; a natural consequence would be reduced infectivity of progeny virus containing envelope proteins misfolded in the presence of these inhibitors. Accordingly, to determine the effect of treatment on progeny virion infectivity, the effect of 16F16, PACMA31, and auranofin treatment on the CHIKV genome:PFU ratio was investigated as a proxy for total-particle:infectious-particle ratio (EN460 was excluded due to its relatively high EC_{log50} at early time points). In 3 independent experiments (n = 3/treatment group per experiment), HEK293 cells were treated with 10 μM PACMA31 or 16F16, 1 μM auranofin, or 1% DMSO for 1 h prior to infection with an MOI of 2 PFU/cell CHIKV-YO123223, and supernatant and cell lysates were collected 8 HPI. Infectious particles in the supernatants were quantified by plaque assay, while RNA was isolated using Direct-Zol RNA extraction (Zymo Research, Irving, CA) and quantified by RT-qPCR using the Taqman RNA-to-Ct one-step kit following manufacturer's instructions (LifeTechnologies, Carlsbad, CA) using previously described primers/probe (Erasmus et al., 2017). 16F16 and auranofin treatment resulted in an increase in the genome:PFU ratio (Kruskal-Wallis and pairwise comparison with Bonferroni correction, $p < 0.05$; Fig. 3A), consistent with an increase in non-infectious particles. Treatment with 16F16 and PACMA31 resulted in similar levels of viral RNA in cell lysates, while auranofin-treated cells had less intracellular viral RNA (Kruskal-Wallis and pairwise comparison with Bonferroni correction, $p < 0.05$; 3B). This result was consistent with infected cells releasing their contents, including viral genomes and/or misfolded/incompletely assembled particles, into the supernatant due to apoptosis or other cell death processes.

Additionally, to ascertain the effect of auranofin and 16F16 on E1

Table 1
CC_{50}, EC_{log50}, and TI values for PDI, TRX-R, and ERO-1 inhibitors against CHIKV in HEK293 cells.

Treatment	Measure	Hours post-infection			
		8	12	24	48
16F16	EC_{log50} (μM) [Err.]	1.0 [0.52]	3.0 [0.14]	4.5 [0.09]	6.6 [0.45]
	CC_{50} (μM) [Err.]	12.2 [10.1]	12.0 [10.3]	9.7 [9.8]	8.9 [9.2]
	TI	12.2	4	2.14	1.35
PACMA31	EC_{log50} (μM) [Err.]	2.8 [0.51]	3.9 [0.10]	10.3 [0.33]	12.1 [0.31]
	CC_{50} (μM) [Err.]	57.9 [10.6]	22.4 [8.9]	13.4 [6.9]	12.2 [9.7]
	TI	20.68	5.74	1.30	1.00
EN460	EC_{log50} (μM) [Err.]	10.2 [0.30]	15 [0.29]	22.5 [0.34]	27.0 [0.24]
	CC_{50} (μM) [Err.]	94.1 [15.2]	40.2 [20.8]	32.5 [20.8]	31.1 [7.6]
	TI	9.23	2.64	1.44	1.15
Auranofin	EC_{log50} (μM) [Err.]	3.2 [0.62]	0.1 [0.5]	0.4 [0.33]	1.0 [0.13]
	CC_{50} (μM) [Err.]	5.85 [10.0]	10.45 [14.9]	7.5 [8.8]	1.6 [8.6]
	TI	1.83	104.5	18.75	1.6

EC_{log50}, 50% log_{10} reduction of viral titer; CC_{50}, 50% reduction in cell viability; TI, therapeutic index (CC_{50}/EC_{log50}); [Err], error of the fit of the overall curve.