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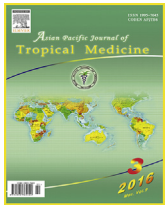

Original research http://dx.doi.org/10.1016/j.apjtm.2016.01.033

# Pharmacodynamics of aminoglycosides and tetracycline derivatives against Japanese encephalitis virus

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ARTICLE INFO

*Article history:*
Received 15 Dec 2015
Received in revised form 20 Dec 2015
Accepted 30 Dec 2015
Available online 13 Jan 2016

*Keywords:*
Japanese encephalitis virus
2 DOS aminoglycoside
Doxycycline
Kanamycin

ABSTRACT

**Objective:** To explore the antiviral activity of antibiotic compounds, mainly aminoglycosides and tetracyclines against Japanese encephalitis virus (JEV) induced infection *in vitro*.

**Methods:** Antiviral activity were evaluated against JEV using cytopathic effect inhibition assay, virus yield reduction assay, caspase 3 level, extracellular viral detection by antigen capture ELISA and viral RNA levels.

**Results:** JEV induced cytopathic effect along with reduction of viral progeny plaque formation indicated antiviral potential of the compounds suggesting that antibiotics had broad spectrum activity. Doxycycline and kanamycin administration in dose dependent manner declined viral RNA replication.

**Conclusions:** The present study shows kanamycin and doxycycline can affect virion structure and alter replication causing inhibition of JEV induced pathogenesis *in vitro*.

## 1. Introduction

Japanese encephalitis (JE) is an arboviral neurologic disease of global public health importance. The disease is endemic in many parts of South Asia, Southeast Asia, East Asia and the Pacific. Most of JE disease is asymptomatic with no apparent illness. The ratio of asymptomatic to symptomatic infection varies between 25:1 and 1000:1 [1]. Case fatality rate among symptomatic patients is 25%–30%, with neuropsychiatric sequelae evident in 30%–50% of survivors [2]. Annually, approximately 30000–50000 JE cases, including 10000 deaths are reported [3]. JE is caused by a positive sense RNA virus–Japanese encephalitis virus (JEV), a member of JEV serocomplex of genus *Flavivirus* under family *Flaviviridae*. JEV is transmitted in humans mainly by *Culex* species of mosquitoes [4]. The treatment therapy for JE is mainly conservative and supportive.

Recently, tetracyclines and aminoglycosides specifically 2 deoxy streptamine (2 DOS) aminoglycoside derivative compounds have been successfully proved beneficial against viral infection namely dengue virus, West Nile virus and reovirus [5–7]. We report the efficiency of doxycycline and kanamycin against JEV induced infection in an *in vitro* pharmacokinetic model system.

## 2. Materials and methods

### 2.1. Biosafety statement

All infectious work with JEV was performed in a high containment facility at Arbovirology Laboratory, Regional Medical Research Centre (RMRC), Northeast Region, Indian Council of Medical Research, India. The present work was done under the Senior Research Fellowship scheme awarded by Indian Council of Medical Research, India.

### 2.2. Cell line, drugs and virus

Baby hamster kidney (BHK-21), a fibroblast cell line was procured from National Cell Centre for Sciences, Pune, India and maintained at RMRC, Northeast Region. The cell line was being maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 5% tryptose phosphate broth. Eight drugs were used in the present study: brefeldin A, chlortetracycline, demeclocycline, doxycycline, gentamicin, kanamycin, rolitetracycline and tetracycline.

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Peer review under responsibility of Hainan Medical College.

Ribavirin, a nucleoside analog metabolite compound and minocycline, a second generation tetracycline compound were used as control drugs. JEV strain P20778 (GenBank accession no. AF080251, source: human, year: 1958, place: Vellore, India) was obtained from National Institute of Virology, Pune, India and propagated in BHK-21 cell line.

### 2.3. Cytotoxicity of antibiotics and standards

Initially, cytotoxic concentration at 50% end point ($CC_{50}$) for the drugs were determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay [8,9]. Briefly, a 96 well plate (made by Genaxy) was pre-seeded with $3.5 \times 10^5$ cells/mL overnight. Next day, the MEM was discarded and replaced with respective dose concentration of drugs starting from 0 μg/mL to 520 μg/mL prepared in MEM. In control wells, plain MEM was added and allowed to incubate for 72 h (37 °C, 5% $CO_2$). After incubation, MTT reagent was added under dark conditions. Plate was again incubated for 4 h. After this, MTT solvent solution was added and incubated overnight. Following day, the reading of the plate was taken at 490 nm in an ELISA reader [10]. The optical density reading of the drugs at different concentrations was plotted. The concentration of compound reducing cell viability by 50% ($CC_{50}$) was determined from a curve relating percent cell viability to the concentration of compounds.

### 2.4. Screening for virus induced cytopathic effect inhibition (CPEI)

Since the two drugs, brefeldin A and demeclocycline, showed high cytotoxicity at very low doses, subsequent experiments were carried out with remaining drugs. Antiviral activity assay of drugs against JEV strain P20778 were screened using CPEI assay in a 96 well plate [8].

### 2.5. Confirmation of antiviral activity by virus yield reduction assay

The drug susceptibility was evaluated by virus yield reduction assay. Cells were pre-treated with different concentrations of drugs (0–100 μg/mL) for 24 h prior to infection. BHK 21 cells were inoculated with JEV strain P20778 at a multiplicity of infection of 0.1. Following a 1 h virus incubation period, the medium was removed and infected cultures were incubated with medium containing respective concentrations of drugs. At 48 h post-infection cultures were deep-frozen at −80 °C. After thawing of the cultures, infectious virus titres were determined for progeny virus yields by standard plaque titrations. All the experiments were run in triplicates. Percentage inhibitions of plaques were determined using the following formula:

% Inhibition = (Number of plaques in virus control − Number of plaques in drug treated)/(Number of plaques in virus control) × 100

The antiviral activity was expressed as 50% inhibitory concentration ($IC_{50}$) of the compound, *i.e.* concentration of the compound required to inhibit viral plaques by 50% as compared to virus control [6,8,11].

### 2.6. Extracellular antigen detection by Ag capture ELISA

An Ag capture ELISA was standardized to detect presence of antigens based on Gajanana *et al.*, 1995 with modifications [12].

### 2.7. Infectious virus yield inhibition by $TCID_{50}$/mL

The supernatant fluid was used to determine virus yield based on Reed and Munch method [13].

### 2.8. Caspase 3 assay: indicators of apoptosis activation

Caspase 3 of the samples was evaluated by using Caspase 3 Assay Kit, Colorimetric (made by Sigma–Aldrich company) according to manufacturer's instructions.

### 2.9. Monitoring of JEV RNA loads

*JEV* RNA was monitored using primers: F 5′-GGGAGTGATGGCCCCTGCAAAATT-3′ and R 5′-TCCAATGGAGCCAAAGTCCCAGGC-3′ [14]. The specificity of the amplicon was verified by melt curve analysis. Viral RNA load in antibiotics treated cells was compared to untreated controls and was normalized to the reference gene (*β-actin*). *β-Actin* gene primers for BHK-21 were: 5′-ACTGGCATTGTGATGGACTC-3′ and 5′-CATGAGGTAGTCTGTCAGGTC-3′ [15]. Data was expressed as relative fold expression to untreated controls, which was defined as 1.0 fold (100%). Triplicate reactions were carried out for each sample and no template control was included as a negative control.

### 2.10. Statistical analysis

Statistical analysis was done with the help of SPSS v.16 software. $CC_{50}$ and $IC_{50}$ values were calculated using means with standard deviations (SD). An unpaired student's *t* test was used for comparisons between 2 groups. One way ANOVA test was used to determine significance among the groups. Value of $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Antiviral screening of drug efficacy

Initially, $CC_{50}$ of all 10 drugs were determined (Table 1). Out of all drugs, both brefeldin A and demeclocycline showed

**Table 1**
$CC_{50}$, $IC_{50}$ and TI dose of candidate compounds.

| Candidate compound | $CC_{50}$ (μg/mL) | $IC_{50}$ (μg/mL) | TI |
|---|---|---|---|
| Brefeldin A | 10 ± 2 | – | – |
| Chlortetracycline | 189 ± 8 | 50 ± 7 | 4.0 |
| Demeclocycline | 9 ± 2 | – | – |
| Doxycycline | 95 ± 6 | 22 ± 1.2 | 4.3 |
| Gentamicin | 109 ± 12 | 53 ± 9 | 2.0 |
| Kanamycin | 381 ± 139 | 70 ± 15 | 5.4 |
| Rolitetracycline | 300 ± 77 | 76 ± 25 | 4.0 |
| Tetracycline | 303 ± 139 | 74 ± 33 | 4.0 |
| Standard | | | |
| Minocycline | 342 ± 17 | 34 ± 9 | 10.0 |
| Ribavirin | 195 ± 4 | 20 ± 2 | 10.0 |

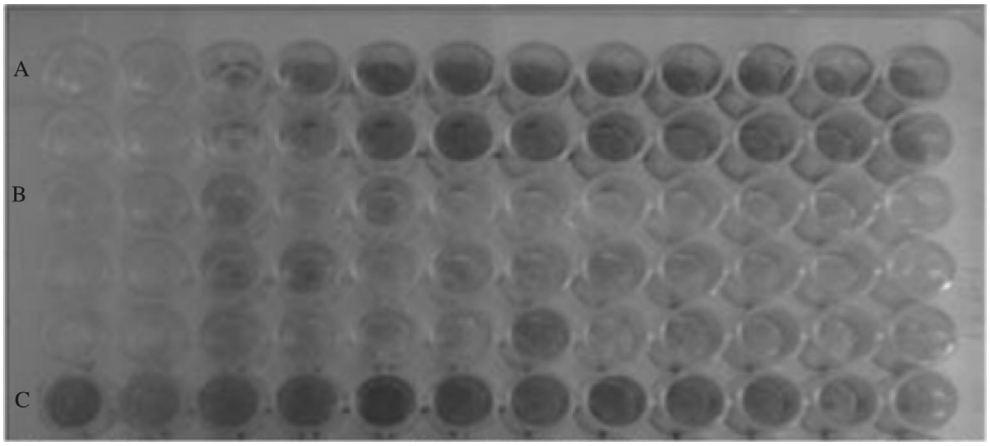


**Figure 1.** CPEI assay of doxycycline against P20778 strain.
A: virus + drug doxycycline treated cells; B: virus treated cells; C: cell control.

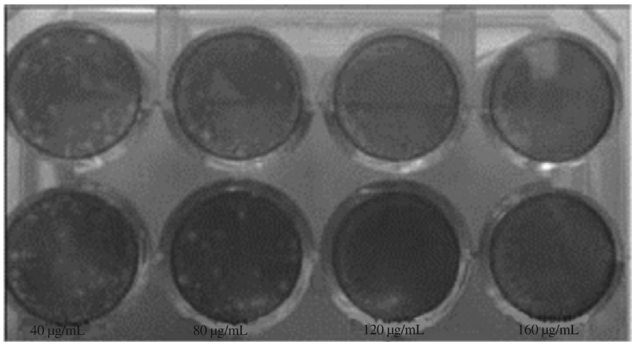


**Figure 2.** Virus reduction assay for drug chlortetracycline determining $IC_{50}$ of chlortetracycline.

cytotoxicity at minimum concentration range. Therefore, these 2 drugs were excluded from further experiments. BHK-21 cell line was used to study JEV induced pathogenicity due to their susceptibility to JEV entry and replication. The effect of the experimental drugs upon *in vitro* JEV induced infection was screened. The antiviral activity of the drugs was initially evaluated using CPEI assay. JEV infected BHK-21 cell line exhibited a typical cytopathic effect (CPE) with detached rounded cell bodies. It was observed that all 8 drugs had

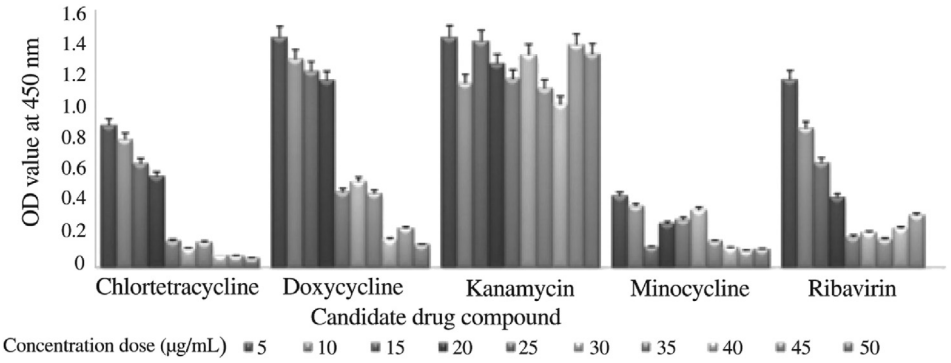


**Figure 3.** Extracellular viral antigen post treatment with candidate drug compounds.

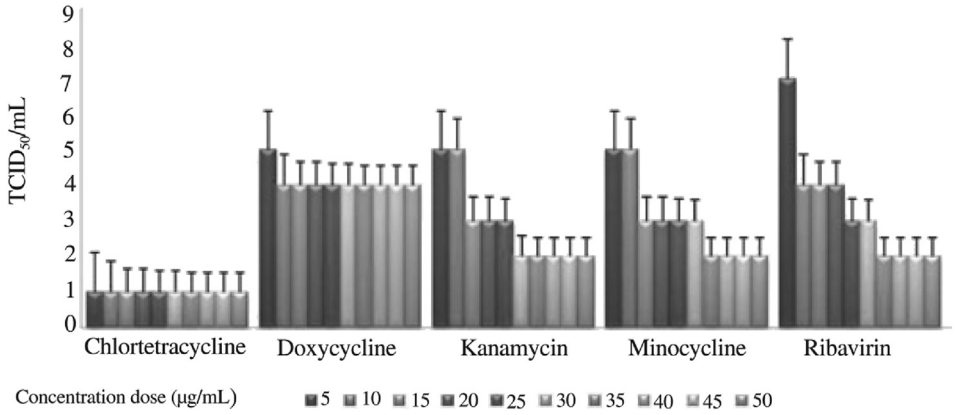


**Figure 4.** Progeny virus yield post treatment with candidate drug compounds.

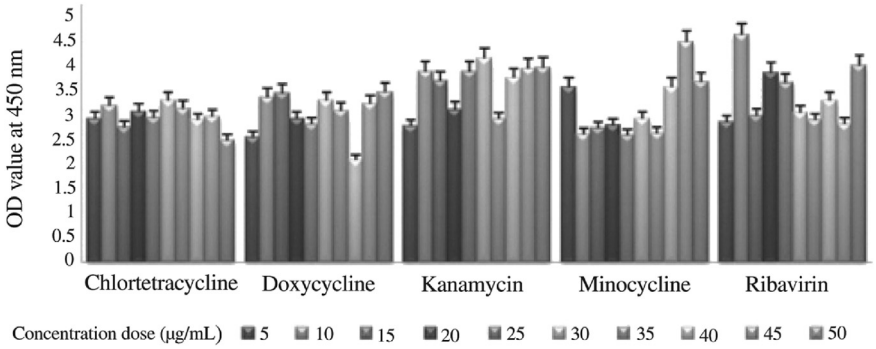


**Figure 5.** Caspase 3 levels at 48 h post infection and treatment with candidate compounds.

exhibited JEV induced CPE reduction in a concentration dependent manner (Figure 1). The compounds subjected to virus yield reduction showed a dose dependent reduction of plaques formed by JEV strain P20778 with an $IC_{50}$ value of kanamycin, chlortetracycline (Figure 2) and doxycycline of (70 ± 15) μg/mL, (50 ± 7) μg/mL and (22 ± 1.2) μg/mL respectively (Table 1). The specificity of antiviral compound was determined by calculating therapeutic index (TI), which was a ratio of $CC_{50}$ to $IC_{50}$ (Table 1). Kanamycin (TI: 5.4), doxycycline (TI: 4.3) and chlortetracycline (TI: 4) showed moderate activity against JEV compared to the two standards. The standards, minocycline and ribavirin exhibited high TI. Therefore, subsequent experiments were carried out with kanamycin, chlortetracycline and doxycycline.

### *3.2. Kinetics of antiviral activity of drugs against viral antigen and viral progeny yield*

The antiviral activity of drugs was evaluated by presence of viral antigen ($P < 0.05$) (Figure 3) and inhibition of virus yield ($P < 0.05$) (Figure 4). It was seen that doxycycline and kanamycin showed decline of soluble JEV antigen and infectious virus yield progeny on dose dependent manner.

### *3.3. Drug inhibition on JEV induced caspase 3 activated apoptosis pathway*

It was observed that chlortetracycline, doxycycline and kanamycin did not have any effect on extracellular caspase 3 protein reduction ($P < 0.05$) (Figure 5).

### *3.4. JEV viral RNA inhibition monitored by qPCR*

Viral RNA was inhibited at concentration dependent manner. Doxycycline (5–40 μg/mL), kanamycin (5–50 μg/mL) and chlortetracycline (10–50 μg/mL) showed viral RNA inhibition at varied drug concentration range. Standard minocycline showed viral RNA inhibition at 20–50 μg/mL dose concentration (Figure 6).

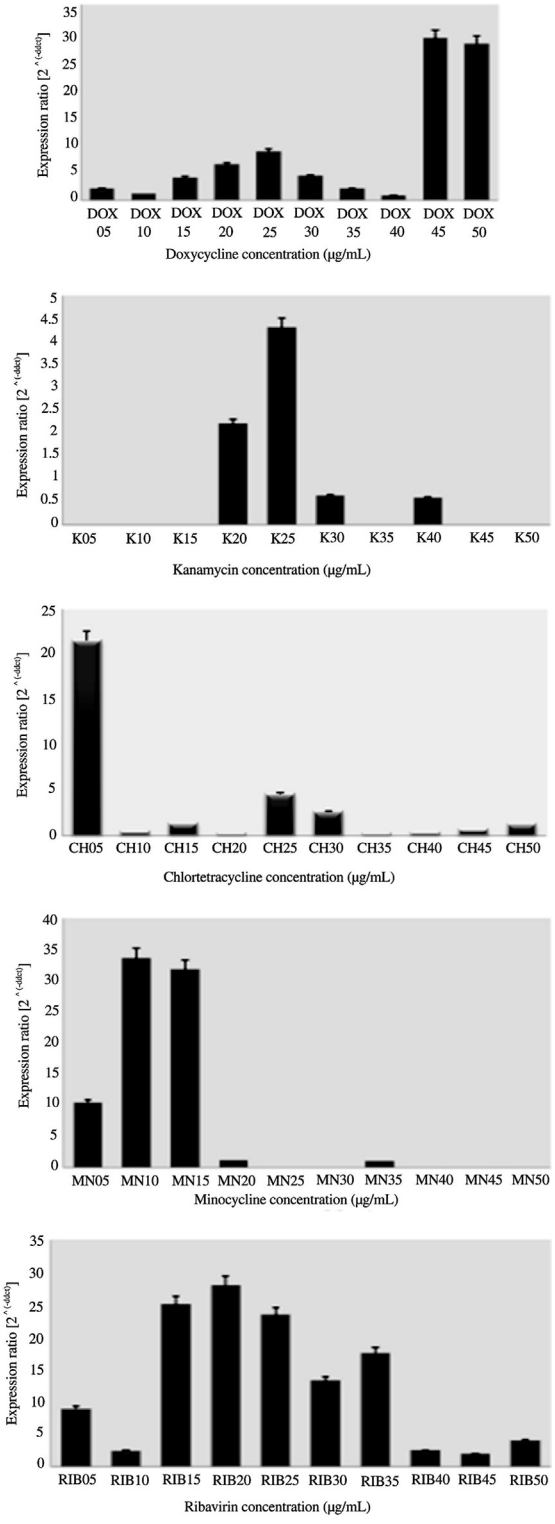


**Figure 6.** Expression ratio at concentrations (5–50 μg/mL) of candidate drug compounds.

## 4. Discussion

This study is set to identify synthetic compounds belonging to tetracyclines and 2 DOS aminoglycosides groups as effective therapeutic agents against JEV in an *in vitro* system. Antibiotics are effective against bacterial microbes with the main target sites include: the decoding (or A-site) on the 30S, the peptidyl transferase center on the 50S, and the peptide exit tunnel on the 50S of ribosomes [16]. However, in recent years, certain broad spectrum antibiotics having protein synthesis inhibition mode of action, have also been found to be effective against viral etiologies [17,18]. *In vitro* susceptibility of viruses to antiviral agents is often assessed on ability to lower virus induced CPE [8]. Inhibition of JEV induced CPE suggests that antibiotics have broad spectrum activity. In the present study, antiviral effect of antibiotics, as witnessed in the virus yield reduction assay with reduction of viral progeny plaque formation, was another pointer of antiviral potential of the compounds. However, $IC_{50}$ values of these three drugs were relatively higher but they all fell within the range of optimum drug concentration recommended for use in cell culture [19,20]. Antiviral activity against JEV was exhibited by kanamycin, doxycycline and chlortetracycline with TI values of 5.4, 4.3 and 4.0 respectively. We demonstrated that treatment of cells with antibiotics greatly reduced yield of JEV at sub-cytotoxic dose. Thus, we can state that the effects of drugs were in direct action with the virus and not affecting the host cell membrane.

JEV induces apoptosis of infected cells. Caspase 3, the executor molecule of apoptotic signaling pathway is activated in late stage of cell death programme. However, the drugs were unable to restrict the caspase 3 level, thus showing no role in inhibition of extracellular caspase 3 protein molecules.

Viral progeny decreased as evidenced by plaque assay, showed that the drugs inhibited JEV replication in the cell line. Drugs at dose dependent manner declined viral RNA replication, especially doxycycline and kanamycin, which showed similar results with standard drug, minocycline [21]. Both the drugs, doxycycline and kanamycin exhibited significant decline in viral RNA load in the drug treated cells. *In vitro* findings are also supported by the *in silico* study, where, virtual screening of JEV NS3 helicase/NTPase and candidate drugs (mainly kanamycin and doxycycline) showed favorable interactions in motifs I, II and VI of helicase suggesting possible viral replication inhibition (RMRC unpublished data).

Kanamycin belongs to 2 DOS aminoglycosides antibiotics compounds. 2-DOS contains a rigid framework of hydrogen bond donors among which the rigid cis-1, 3 arrangements of amino groups is responsible for selective interaction with structural motifs in RNA targets. The primary site of mode of action of 2 DOS aminoglycosides is 30S of bacterial ribosomes. Recently, other sites of drug action have been discovered, which are self splicing group I introns, hammerhead ribozyme, hepatitis delta ribozyme, RNAse P and transfer RNA. Multiple aminoglycosides binding sites have been reported on both small and large RNA molecules. Aminoglycosides have also shown to bind to hepatitis viral RNA, blocking formation of RNA-peptide and RNA-protein complex. 2 DOS aminoglycosides are potential viral RNA binding molecules in case of human immunodeficiency virus which is a single stranded positive sense enveloped virus. There are multiple RNA–RNA and RNA-protein interactions in human immunodeficiency virus life cycle, some of which may serve as potential targets for aminoglycosides based therapeutics based on aminoglycoside scaffolds. Therefore, it can be stated that aminoglycosides are rather universal binders which binds to different RNA targets. The possible mode of interaction may be the electrostatic interactions between RNA and aminoglycoside moieties which probably cause 'structural electrostatic complementarities' [17]. This mechanism probably explains the ability of kanamycin acting against JEV, also a single stranded positive sense RNA virus.

Polycyclic naphthacene carboxamide derived compounds or more commonly known as the tetracycline group of compounds are protein synthesis inhibitor molecules acting on bacterial microbes. There are numerous non bacterial uses of tetracycline compounds. Hydroxyl groups in tetracycline rigid skeleton are a source of radical oxygen species that irreversibly damage macromolecules such as DNA, RNA and proteins. These effects lead in cellular death for oxidative stress. The compounds alter balance equilibrium sequestering divalent ions (ex-$Ca^{2+}$) much more than the monovalent ions (ex-$K^{+}$) in eukaryotic cells. Tetracycline forms complexes with $Ca^{2+}$ and $K^{+}$ ions present in blood plasma. Highly lipophilic tetracycline compound like doxycycline exhibits bactericidal mechanism, which causes membrane damage (as ionophores) [18]. Another compound chlortetracycline is a potent and specified $Ca^{2+}$ ionophore [22]. Therefore, tetracyclines have been recently identified as a family containing chemically diverse mechanisms of activity which interacts with multiple targets.

Thus, we can state that tetracyclines and aminoglycoside compounds have diverse mode of action against non conventional target microbes. We have found in our present study the efficacy of kanamycin, a 2 DOS aminoglycoside and doxycycline, a second generation tetracycline compound affecting virion structure and altering replication causing inhibition of JEV induced pathogenesis in an *in vitro* pharmacokinetic model system.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgments

The authors are grateful to Dr. Jani Borah for continuous support and helpful discussions during course of the experiments. Rashmee Topno is supported by ICMR SRF programme, India.

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