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# Broad-spectrum inhibitor of viruses in the *Flaviviridae* family

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**Abstract**

The viruses in the *Flaviviridae* family have been associated with human and animal diseases. In this report, we demonstrate that compound 2-amino-8-(β-D-ribofuranosyl) imidazo [1,2-a]-s-triazine-4-one (ZX-2401) was capable of inhibiting the production in culture of at least five members of the *Flaviviridae* family with minimal cytotoxicity. This compound inhibited yellow fever virus, dengue virus, bovine viral diarrhea virus, banzi virus and West Nile virus with $EC_{50}$ of 10, 10, 5, 5 and 3 μg/ml, respectively, and the $CC_{50}$ in these experiments were greater than 1000 μg/ml. The activity of ZX-2401 is comparable to or better than the control drugs in these studies and was not affected by MOI variation. In addition, ZX-2401 inhibited HCV replication in a dose response fashion in the replicon assay system. Furthermore, ZX-2401 exhibited a synergistic antiviral activity in combination with IFN in tissue culture. The data described herein suggest that ZX-2401 is a broad-spectrum inhibitor of the RNA viruses, which has merit for development of treatments for the emerging infections caused by the viruses in the *Flaviviridae* family.


*Keywords:* Antivirals; Flavivirus; West Nile virus; Hepatitis C virus; Nucleoside analog; Broad-spectrum inhibitor

## 1. Introduction

The *Flaviviridae* are a family of at least 66 viruses, of which almost half have been associated with human disease. The most well-known are hepatitis C virus (HCV), dengue fever virus, yellow fever virus, West Nile virus and Japanese encephalitis. In addition, flaviviruses also cause disease in domestic or wild animals of economic importance.

HCV infection is the most common chronic blood-borne infection in the United States. There are about 36,000 new infections every year, of which 25–30% are symptomatic. It is estimated that 3.9 million (1.8%) Americans have been infected (Alter, 1995; Alter et al., 1992; Barrera et al., 1995; NIH, 1997; Prince et al., 1993; Thomas et al., 1995). The mosquito-borne flavivirus, dengue, is estimated to cause 100 million cases of dengue fever, 500,000 cases of dengue hemorrhagic fever and 25,000 deaths each year with 2.5 billion people at risk worldwide (Monath, 1994). West Nile virus (WNV) is the causative agent of West Nile (WN) fever. The common complication is encephalitis (Huang et al., 2002). WN fever is a mosquito-borne flavivirus infection that is transmitted to vertebrates primarily by various species of *Culex* mosquitoes. Like other members of this serogroup of flaviviruses, WNV is maintained in a natural cycle between arthropod vectors and birds. The first known human case of WNV infection recorded in the Western Hemisphere was reported in August 1999 (CDC, 1999); eventually, 62 cases of the disease were later confirmed (CDC, 2000). This outbreak was concurrent with increased mortalities among birds and horses. Initially, 70% of the human laboratory-confirmed cases occurred within a 10-km radius, centered in the northern end of the New York City borough of Queens (CDC, 1999); however, recent reports have shown that this virus has persisted over the years in the United States. It has spread to other states on the eastern seaboard during 2000 and 2001, suggest-

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ing that WNV is now endemic in the United States and that its geographic range probably will continue to expand until it extends over much of the continent (CDC, 2001). In 2002 and 2003, the spread of WNV reached epidemic proportion (CDC, 2002, 2003).

Although a successful vaccine against the prototypical flavivirus, yellow fever virus, has been in use since the 1930s, and vaccines to two other flaviviruses, Japanese encephalitis virus and tick-borne encephalitis virus, are currently available, at this time there are no vaccines approved for dengue fever, WN infection and HCV infection.

In addition, ribavirin, which is used in combination with interferon (IFN) as the first-line therapy for many of the viruses in this family, has added an additional toxic side effect to the treatment (Markland et al., 2000). The side effect of ribavirin is anemia, which results from the accumulation of the triphosphate form of the drug in erythrocytes. As a result, there is a great need to develop new compounds to be used alone or in combination with IFN to improve efficacy and safety in patients infected with these viruses. To this end, we have identified a compound, 2-amino-8-(β-D-ribofuranosyl) imidazo [1,2-a]-s-triazine-4-one (ZX-2401), which possesses broad-spectrum antiviral activity. The compound designated as ZX-2401 was originally synthesized and tested against picornaviruses (Kim et al., 1978). This investigation showed that the compound was markedly active against vesicular stomatitis, coxsackie B-1 virus and Echo-6 virus, and moderately active against five rhinoviruses (Kim et al., 1978). The results reported in the Kim et al. study provided strong incentive to evaluate this compound against other RNA-type viruses, including those belonging to the *Flaviviridae* family. In this report, we show that ZX-2401 demonstrated comparable activity to ribavirin against yellow fever virus (YFV) and bovine viral diarrhea virus (BVDV), but was superior to ribavirin against banzi virus (BV), dengue virus (DV) and West Nile virus (WNV). The broad-spectrum activity exhibited by ZX-2401 against these viruses demonstrate its suitability for further evaluation as a therapeutic agent against infection brought about by the viruses in the *Flaviviridae* family.

## 2. Materials and methods

### 2.1. ZX-2401

The material used in the preliminary experiment was a gift from Dr. Ganapathi R. Revankar to Dr. Joshua O. Ojwang. The subsequent material used for most of the studies described was freshly prepared as described below.

### 2.2. Preparation of ZX-2401

The preparation was accomplished by the synthesis scheme previously reported by Kim et al. (1978), starting from commercially available cyanuric chloride. Briefly, selective amination of cyanuric chloride **1** with gaseous ammonia at 0 °C followed by careful hydrolysis of one of the halogens of **2** gave 2-amino-4-chloro-6-hydroxy-1,3,5-triazine 3. Reaction of **3** with aminoacetaldehyde dimethyl acetal in aqueous basic media at reflux temperature furnished the intermediate **4**. The acetals groups were hydrolyzed using 6N hydrochloric acid followed by ring annulation in concentrated sulfuric acid at 95 °C and gave crystalline 2-aminoimidazo[1,2-a]-s-triazine-4-one **6** (5-aza-7-deazaguanine). Glycosylation was achieved by first converting **8** to its trimethylsilyl derivative in HMDS followed by treatment of this intermediate with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose in anhydrous dichloroethane in presence of stannic chloride. The reaction was stereoselective and on purification gave only the β anomer. The protecting groups were removed in sodium methoxide in methanol and on recrystallization of the resulting crude product gave a good yield of 2-amino-8-(β-D-ribofuranosyl) imidazo [1,2-a]-s-triazine-4-one **8** (5-aza-7-deazaguanosine) (Fig. 1).

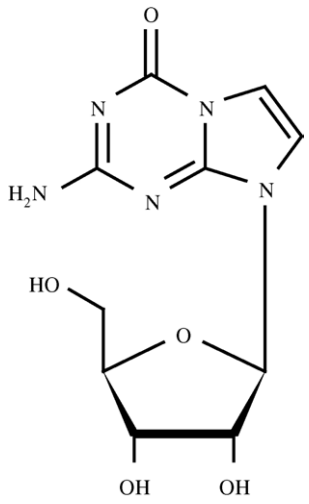


Fig. 1. 5-Aza-7-deazaguanosine (ZX-2401).

The compound is a white powder, which was dissolved in water at 10 mg/ml to make a stock solution. The working solutions were prepared by diluting the stock solution in culture medium to appropriate concentrations needed for each assay. Ribavirin and IFN alpha B/D were from Ribopharm (a division of ICN Pharmaceuticals, Costa Mesa, CA). Units of IFN that were used were based upon the titer provided by Ribopharm for the full strength material (in international units/ml). A unit of IFN is defined as the amount causing a 50% reduction in the CPE of vesicular stomatitis virus in L929 cell culture.

### 2.3. Viruses

Banzi virus (BV), H 336 strain, was purchased from the America Type Culture Collection (ATCC), Manassas, VA. It was isolated from serum of a febrile boy in South Africa. Pools of the virus were prepared in Africa green monkey

(Vero) cells. Vero cells were used for antiviral and cytotoxicity testing.

Dengue virus (DV) type 2, New Guinea strain, was obtained from the Centers for Disease Control and Prevention (CDC), Fort Collins, CO. Pools of the virus were prepared in Vero cells. MA-104 cells (another African green monkey kidney cell line) were used for antiviral testing.

Bovine viral diarrhea virus (BVDV), TN131 strain, was obtained from Blair Fujimoto of Hyclone Laboratories, Logan, UT, who obtained the virus from John Black, American BioResearch, Milton, TN. It was originally obtained from the spleen of a calf with diarrhea. Pools of the virus were made up in bovine turbinate (BT) cells. BT cells were used for antiviral testing.

Yellow fever virus (YFV), 17D strain, was obtained from ATCC. It was originally prepared from infected mouse brain. Pools of the virus were prepared in Vero cells. Vero cells were used for antiviral testing.

Two strains of WNV used were strain B956 (ATCC VR-82; ATCC, Manassas, VA) and a New York isolate from homogenized crow brain (NY, CDC 996625, V1 D3 11/10/1999, Robert Lanciotti, CDC, Division of Vector-Borne Infectious Diseases, Fort Collins, CO).

### 2.4. Cells and media

The following cells and media were used with the appropriate virus: BT cells were obtained from ATCC. Growth medium was Eagle's minimum essential medium with non-essential amino acids (MEM), 10% fetal bovine serum (FBS) and 0.1% $NaHCO_3$ and 50 μg gentamicin/ml.

MA-104 cells were obtained from Whittaker MA Bioproducts, Walkersville, MD. Growth medium was MEM 199, 5% FBS, 0.1% $NaHCO_3$ without antibiotics. Test medium for DV was MEM, 2% FBS, 0.18% $NaHCO_3$ and 50 μg gentamicin/ml.

Vero cells were obtained from ATCC. Growth medium was MEM 199, 5% FBS, 0.1% $NaHCO_3$ without antibiotics. Test medium for BV and YFV was MEM, 2% FBS, 0.18% $NaHCO_3$ and 50 μg gentamicin/ml.

For WNV, African green monkey kidney cells (Vero 76, ATCC CCL1587) were used. MEM with 1% FBS, 0.1% $NaHCO_3$ and 50 μg/ml gentamicin (Sigma, St. Louis, MO) were used to maintain cells during antiviral experiments. Virus stocks were prepared in MA104 cells and stored at −80 °C. The viruses were titered in lightly confluent Vero cells in 96-well microtiter plates.

### 2.5. Assay systems

The assay systems used in the experiments discussed in this report are described below.

#### 2.5.1. Antiviral testing using CPE assay

The CPE assay was performed as previously described by Smee et al. (1988). Briefly, the compounds were diluted in the same type of medium used to prepare the compounds, and appropriate cell types for each target virus were incubated overnight at 37 °C in order to establish a cell monolayer. When the monolayer was established, the growth medium was decanted and various dilutions of test compound were added to each well (6 wells/dilution, 0.1 ml/well). Compound diluent medium was added to cell control wells and virus-only control wells (0.1 ml/well). Virus, diluted in test medium, was then added to appropriate wells at 0.1 ml/well approximately 5 min after the compound. Test medium without virus was added to all toxicity control wells (2 wells/dilution of each test compound) and to cell control wells at 0.1 ml/well. The plates were sealed with plastic wrap (Saran®) and incubated at 37 °C in a humidified incubator with 5% $CO_2$, 95% air atmosphere, until the virus control wells had adequate cytopathic effect (CPE) readings. This was usually achieved after 72 h. Cells were then examined microscopically for virus-induced CPE, which was scored from 0 (normal cells) to 4 (maximal, 100%, CPE). The cells in the toxicity control wells were observed microscopically for morphologic changes attributed to cytotoxicity at the same time. The cytotoxicity was also graded at T (100% toxicity, complete cell sloughing from plate), PVH (80% cytotoxicity), PH (60% cytotoxicity), P (40% cytotoxicity), PS (20% cytotoxicity) and 0 (normal cells.). The 50% effective concentration ($EC_{50}$) and 50% cytotoxic concentration ($CC_{50}$) were calculated by regression analysis of the virus CPE data and the toxicity control data, respectively. The selectivity index (SI) for each test compound was calculated by the formula $SI = CC_{50}/EC_{50}$.

#### 2.5.2. Neutral red testing procedures

The neutral red evaluation was conducted as previously described by Player et al. (1998). Briefly, the plates were first read visually for cytopathology and toxicity, after which 0.1 ml of sterile neutral red (0.034% physiological saline solution) was added to each well. The plates were wrapped in aluminum foil to eliminate light exposure and placed at 37 °C for 1–2 h. All medium was removed, and the cells were washed twice (0.2 ml/well for each wash) with phosphate buffered saline. The plates were inverted and allowed to drain on a paper towel. Neutral red was extracted from the cells by adding 0.2 ml of an equal volume mixture of absolute ethanol and Sörensen citrate buffer, pH 4, to each well and placing the plates at 540 nm with a Model EL309 microplate reader (Bio-Tek Instruments Inc., Winooski, VT). The $EC_{50}$ and $CC_{50}$ were calculated by regression analysis. The SI for each compound tested was calculated using the formula: $SI = CC_{50}/EC_{50}$.

#### 2.5.3. Virus yield reduction (VYR) assay

To delineate the actual antiviral effects of test compounds from the potential cytotoxic effects of the compounds, the infectious virus recovered from the antiviral assays was quantified using the VYR assay (Smee et al., 1992) for various days of post-infection cultures. The test method as described above for visual inhibition of CPE assay was used; inhibition

of CPE was read visually. The 96-well plate was then frozen at −80 °C and thawed, and the virus from the supernatants was then assayed by using a series of 10-fold dilutions and assaying each in quadruplicate in a monolayer of Vero cells grown in 96-well microplates. Viral CPE was determined visually 6 days later after incubation at 37 °C. The virus titer in relation to the concentration of test compound was plotted to determine a 90% effective concentration ($EC_{90}$), the dose that reduced virus titer by 1 $\log_{10}$.

*2.5.4. HCV replicon assay system*

HCV replicon-containing cells (Blight et al., 2000) obtained from Apath LLC (St. Louis, MO) were plated onto the wells of a 96-well plate at $12 \times 10^3$ cells/well and allowed to adhere for 3 h. Compounds were diluted as specified in complete media before addition to cell monolayers. Human IFN alpha (200 IU/ml) was used as a positive control for decrease of cellular replicon levels. Untreated cells were used as a negative control. After cells were treated for 24 h, total RNA was extracted using the Qiagen 96-well RNeasy kit. All compound concentrations tested, as well as controls, were done in quadruplicate. Replicon RNA was measured using "real-time" quantitative RT-PCR and primers specific for the 3′ NTR of HCV. Amplicon was detected and quantified using syber green fluorescence detection. Data were expressed as threshold cycle number and plotted as a percent of untreated control. The larger the amount of target RNA template present, the fewer cycles were required to reach threshold. Duplicate serial 1:3 dilutions of untreated Ava.5 total RNA were done in the following manner: 1:3, 1:9 and 1:27, and an average taken and plotted on a linear regression curve. All threshold cycle data from the compounds tested were plotted against this curve to obtain percent of untreated control. Non-specific cellular effects of compounds were assessed by measuring glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNA using quantitative RT-PCR with primers specific for GAPDH mRNA.

*2.5.5. Combination experiments*

From previous experiments conducted against YFV, we know that virus is present inside of cells even though virus-induced CPE may be completely reduced. For this reason, the CPE assay alone is insufficient to get good quantitative data for a drug combination study. Thus, the virus yield reduction assay was also employed. This made the experiment a two-part study. In the first part, ZX-2401 and IFN were evaluated for inhibition of viral CPE alone or in combination. Before the start of the combination experiment, both ZX-2401 and the IFN were pre-titrated on cells to find doses reducing viral CPE. ZX-2401 was then used at 320, 100, 32 and 0 μg/ml. IFN was used at 100, 32, 10, 3.2 and 0 units/ml. All possible combinations, in a checkerboard fashion, were made for inhibition of the virus. These combinations were performed in 96-well plates, in a manner similar to that described above. After CPE was determined visually, the plate was frozen at −80 °C until the next day, then thawed. The cell/supernate from three infected wells from each dilution were pooled for virus titer determinations. Each sample was serially diluted in 10-fold increments on new confluent monolayers in 96-well plates, using four wells per dilution. End points were determined by the method of Reed and Muench (1938). Virus titers in each sample were expressed as 50% cell culture infectious doses ($CCID_{50}$) per 0.1 ml. The statistical evaluation was performed on the data using the combination index method described by Schinazi et al. (1982).

## 3. Results

*3.1. Antiviral testing against West Nile virus*

The CPE inhibitory assay described above was used with the following modifications. Serial dilutions of test compounds were added to lightly confluent Vero cells in 96-well microplates, after which 5X $CCID_{50}$ of WNV were added to the cells. Uninfected cells, infected cells with no drug and uninfected drug-treated cells were used as controls. 6-Azauridine (6-aza-U) was used as the control drug. Duplicates of toxicity controls at each drug concentration and triplicates of test samples were performed. After 6 days post-virus exposure, cells were visually scored for CPE. The $EC_{50}$ and $CC_{50}$ were calculated by regression analysis using the means of the CPE ratings at each concentration of the compound.

The results obtained from in vitro evaluation of ZX-2401 and control drug 6-aza-U against two strains of WNV are compiled in Table 1. ZX-2401 showed an excellent activity against both strains of WNV with minimal cytotoxicity to the host cells. The antiviral activity of ZX-2401 was comparable to the control drug. Ribavirin was not used as positive control

Table 1
Effect of ZX-2401 on West Nile virus using neural red assay

| Compound (assay method) | ATCC VR-82 | | | New York isolate | | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ (μg/ml) | $CC_{50}$ (μg/ml) | SI | $EC_{50}$ (μg/ml) | $CC_{50}$ (μg/ml) | SI |
| ZX-2401 (visual) | <4.6 ± 0.02 | >100 | >21 | 9.5 ± 0.07 | >100 | >10.5 |
| ZX-2401 (NR[a]) | 3.0 ± 0.01 | >100 | >33.3 | 8.0 ± 0.13 | >100 | >12.5 |
| 6-Aza-U (visual) | 22 ± 0.65 | 85 ± 2.57 | 3.8 | 0.9 ± 0.006 | 85 | >94.5 |
| 6-Aza-U (NR[a]) | 5.0 ± 0.18 | >100 | >20 | 1.6 ± 0.004 | >100 | >62.5 |

[a] NR, neutral red.

Table 2
Effect of ZX-2401 or 6-azauridine on virus yield reduction (VYR) assay at various time points after initiation of experiment

| Days after experiment initiation | Virus yield reduction assay $EC_{90}$ (μg/ml) | Inhibition of CPE assay $EC_{50}$ (μg/ml) |
|---|---|---|
| ZX-2401 | | |
| Day 2 | 3.3 ± 0.1 | Not determined |
| Day 6 | 40 ± 1.7 | 15 ± 2.0 |
| 6-Azauridine | | |
| Day 2 | 1.6 ± 0.02 | Not determined |
| Day 6 | 431 ± 3.13 | 3.1 ± 0.06 |

in WNV experiments because of its poor activity against this virus in tissue culture (Morey et al., 2002).

To delineate the actual antiviral effects of test compounds from the potential cytotoxic effects of the compounds, virus yield reduction (VYR) assay was performed as described in Section 2. The results obtained from viral yield study are summarized in Table 2. ZX-2401 reduced virus production when measured at day 2-post initiation ($EC_{90}$ = 3.3 μg/ml). The $EC_{90}$ increased 12-fold ($EC_{90}$ = 40 μg/ml) when measured at day 6. This effect of losing antiviral activity at later days into the experiment is similar, but not identical, to that observed with 6-aza-U (Table 2).

To investigate the effect of variation in the MOI on the antiviral activity of ZX-2401, the assays were performed using various MOIs of WNV. The MOI covering 1.5 $\log_{10}$ did not affect the antiviral activity of ZX-2401 (data not shown). This observation is a favorable indicator for an antiviral compound because it suggests that the compound can be utilized over a wide range of viral burden.

### 3.2. Antiviral testing against hepatitis C virus in replicon assay system

Evaluation of ZX-2401 against HCV was conducted and the experiment was carried out at Apath LLC (St. Louis, MO) according the Apath HCV replicon assay protocol. At the same time, the cytotoxicity of the compound was also determined by measuring the effect on GAPDH mRNA.

The results of two separate experiments showed that compound ZX-2401 had a weak anti-HCV activity in the replicon assay; however, the anti-HCV activity was in a dose-responsive manner with an $IC_{50}$ of about 70 μg/ml. At the same time, it exhibited no toxicity as measured by GAPDH mRNA levels (data not shown).

On the basis of the antiviral activity observed with WNV and HCV, further experiments were carried out to investigate the antiviral spectrum of this compound against other viruses the *Flaviviridae* family.

### 3.3. Antiviral testing against yellow fever virus

ZX-2401 was tested against YFV 17D strain and a known positive control drug (ribavirin) was evaluated in parallel with ZX-2401 in each test. After appropriate time post-virus exposure, the plates were scored visually, after which neutral red was added to the medium. The $EC_{50}$ values obtained are presented in Table 3.

Table 3
In vitro effect of ZX-2401 and ribavirin on yellow fever virus, bovine viral diarrhea virus, banzi virus and dengue virus

| Virus | ZX-2401 | | | Ribavirin | | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ (μg/ml) | $CC_{50}$ (μg/ml) | SI | $EC_{50}$ (μg/ml) | $CC_{50}$ (μg/ml) | SI |
| YFV | 10 ± 0.61 | >100 | >10 | 32 ± 3.54 | >100 | >3.125 |
| BVDV | 0.6 ± 0.003 | >100 | >166.6 | 5.0 ± 0.49 | >100 | >20 |
| BV | 5.0 ± 0.36 | >100 | >20 | 60 ± 4.51 | >100 | >1.67 |
| DV | 10 ± 1.76 | >100 | >10 | >80 | >100 | ND |

ND, Not determined.

In this study, ZX-2401 inhibited YFV in cell culture with minimum cytotoxicity. ZX-2401 antiviral activity against YFV was up to three-fold better than ribavirin.

### 3.4. Antiviral testing against dengue virus

Evaluation of ZX-2401 against DV was conducted using CPE assay system described above. As shown in Table 3, ZX-2401 showed excellent activity against DV production in culture. In this experiment, ZX-2401 showed a very superior activity to ribavirin with minimum cellular toxicity. The $EC_{50}$ values were 10 and >80 μg/ml for ZX-2401 and ribavirin, respectively. In addition, ZX-2401 completely inhibited DV production in cell culture at concentration of 32 μg/ml.

### 3.5. Antiviral testing against bovine viral diarrhea virus

Evaluation of ZX-2401 in a pestivirus was conducted using CPE assay against BVDV. The results shown in Table 3 demonstrated that ZX-2401 inhibited BVDV in a dose-dependent fashion, and in this experiment ZX-2401 was almost 10-fold more active than ribavirin.

### 3.6. Antiviral testing against banzi virus

Evaluation of ZX-2401 in a pestivirus was conducted using CPE assay against BV in Vero cells. The results of this experiment show that ZX-2401 was 12-fold more active than ribavirin (Table 3).

### 3.7. Combination experiment

The purpose of this study was to investigate the effects of ZX-2401 and IFN in combination using YFV in cell culture. The data obtained from three separate experiments performed in triplicates is tabulated in Table 4. By itself, ZX-2401 completely reduced viral CPE at 320 and 100 μg/ml, with minimal CPE present at 32 μg/ml. The IFN by itself reduced CPE by 100% at the 100 units/ml dose. There was a dose-responsive effect on CPE reduction between 32 and 3.2 units of IFN. Combinations of ZX-2401 and IFN reduced

Table 4
Effect of combination ZX-2401 and interferon alpha B/D on a yellow fever virus infection in Vero cells, determined by virus titer reduction assay

| ZX-2401 (μg/ml) | Virus titer ($log_{10}$ $CCID_{50}$/0.1 ml) | | | | |
|---|---|---|---|---|---|
| | Interferon (units/ml) | | | | |
| | 100 | 32 | 10 | 3.2 | 0 |
| 320 | 0 ± 0.0[a] | 1.7 ± 0.011[a] | 2.0 ± 0.001[a] | 1.7 ± 0.006[a] | 2.3 ± 0.03 |
| 100 | 1.3 ± 0.002[a] | 4.0 ± 0.14 | 4.7 ± 0.38 | 4.3 ± 0.06 | 3.7 ± 0.04 |
| 32 | 4.7 ± 0.008[a] | 6.3 ± 0.09 | 6.3 ± 0.3 | 5.3 ± 0.02[a] | 5.7 ± 0.2 |
| 0 | 6.0 ± 0.05 | 6.5 ± 0.045 | 6.5 ± 0.37 | 6.3 ± 0.058 | 6.5 ± 0.098 |

[a] Indicates improved results compared to those using ZX-2401 or IFN alone at the same dosages.

viral CPE by 100% at all combinations tested. Toxicity of the compounds alone or in combination was assessed by visual inspection of treated uninfected cultures. No toxicity was evident at any combination or when the compounds were used alone.

ZX-2401 alone reduced YFV titer in a dose-dependent manner. IFN alpha B/D alone may have had a weak effect at 100 units/ml, however, the combination of ZX-2401 (at 320, 100 or 32 μg/ml) and IFN at 100 units/ml reduced virus titers below that of ZX-2401 alone. No other drug combinations appeared to reduce virus titer below that achieved by ZX-2401 alone. Furthermore when evaluated, the combination experiment data in Table 4 using the Combination Index method described by Schinazi et al. (1982), indicated moderate synergistic antiviral effect.

## 4. Discussion

The viruses in the *Flaviviridae* family have recently received attention because of the increased incidences of HCV infection, isolation of WNV in North America and lack of vaccines and cost effective therapies. The isolation of WNV in the Northern Hemisphere in particular has brought awareness that the viruses in this family are not confined to the tropics, and as such, proactive steps are needed to discover and develop therapeutic agents against these viruses.

In this report, we demonstrate that compound ZX-2401 was capable of inhibiting the production in culture of at least five members of the *Flaviviridae* family with minimum cytotoxicity. The activity of ZX-2401 is comparable to or better than the control drugs in these studies. Like ribavirin (Poynard et al., 1998), ZX-2401, if commercially developed, would unlikely be administered by itself as a therapy for flavivirus infection. It is possible that it could be combined with IFN. The fact that there is synergy when these compounds are used in combination provides a significant indication that such combination treatment is possible.

It is noted that ZX-2401 did not lose as much antiviral activity as 6-aza-U at 6 days (Table 2). Several explanations for these findings are possible based on results using 6-aza-U. One explanation is that the drugs were labile over time of incubation on the cells so that with increasing time, they lost efficacy as reflected in the increasing virus titers. Moreover, the CPE might be a delayed response to the initial virus reduction so that CPE-inhibitory effects were not observed until days 4 or 6. This explanation of unstable drug was probably not the cause because addition of fresh 6-aza-U every 2 days did not improve the VYR at day 6 as compared to no addition of fresh compound (data not shown). Another explanation is that the drugs acted as metabolic modifiers and slowed the replication of the virus and consequently the delayed CPE. Over time, however, the virus titers in the 6-aza-U-treated cells reached the same virus levels as the untreated cells. This is consistent with the observation that 100% CPE was observed 8 days post-virus initiation in cells treated with any concentration of 6-aza-U (data not shown). A third possible explanation is that minor populations of virus, not responsive to drug treatment, replicated eventually to high levels to overtake the drug-sensitive variants. It is also recognized that explanations for ZX-2401 may or may not be the same as those for 6-aza-U. Nonetheless, it is important to note that ZX-2401 was approximately 10-fold more active than the control drug in this assay.

The mechanism for the beneficial effect of ribavirin remains unclear given that ribavirin appears not to eradicate viral replication in HCV patients. To date several mechanisms of action (MOA) have been proposed for ribavirin. These include: (a) inhibition of inosine monophosphate dehydrogenase (IMPDH) (Markland et al., 2000); (b) inhibition of proinflammatory mediators induced by viral infection (Ning et al., 1998); (c) inducement of lethal mutagenesis after incorporation during viral RNA synthesis, which leads to loss in total viral genomic RNA (Crotty et al., 2001). The MOA of ZX-2401 is currently unknown; however, based on of the fact that it is also a nucleoside analog with broad-spectrum antiviral activity, it is conceivable that it would exhibit some but perhaps not all MOA that have been proposed for ribavirin. However, unlike ribavirin, ZX-2401 has strong antiviral activity against WNV, implying a different or additional mode of action. This characteristic, coupled with lack of toxicity to the host cells in tissue culture, suggests that it might lack some of the undesirable effects usually associated with ribavirin. Further experiments are needed to elucidate the difference in mechanism(s) of action between ZX-2401 and ribavirin.

The data described herein suggest that ZX-2401 is a broad-spectrum inhibitor of the RNA viruses. The fact that ZX-2401 is less toxic and more active than ribavirin, a compound with

similar chemistry that is widely used to treat these viruses, strongly argues for the development of ZX-2401 to treat infections caused by the viruses in the *Flaviviridae* family as a monotherapy or in combination with other therapies such as IFN.

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