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DEVELOPMENT OF SYSTEMS FOR DELIVERY OF ANTIVIRAL DRUGS

ANNUAL REPORT

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This is our Third Annual Progress Report on Contract No. DAMD17-85-C-5276. It covers research performed during the period September 30, 1987 through September 29, 1988.

Ribavirin, a broad-spectrum antiviral agent with potent activity *in vitro* against a number of important RNA viruses of military significance, is severely limited in its usefulness against virus-induced encephalitic diseases because it does not cross the blood-brain barrier well enough to achieve effective antiviral concentrations in the brain. Our efforts are directed toward the brain-specific delivery of ribavirin and other antiviral agents by means of a redox prodrug concept. The scope of the research program involves the synthesis of CNS-targeted prodrug esters of ribavirin and selenazole, pharmacokinetic studies of drug distribution and sustained delivery of drug in the brain, and the evaluation of the therapeutic efficacy of these antiviral prodrugs compared with the parent drugs in the treatment of lethal Venezuelan equine encephalitis (VEE) virus and Japanese encephalitis (JE) virus infections in mice. In preliminary studies at USAMRIID,

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the initial ribavirin prodrug protected mice from a lethal challenge of JE virus and was much superior in efficacy to the parent drug which had no effect. Attempts to confirm the original observation of *in vivo* efficacy of this ribavirin prodrug at Southern Research Institute were unsuccessful, but differences in virus strain, route of inoculation, and treatment protocol may have accounted for the failure to detect antiviral efficacy. *In vivo* antiviral evaluations were begun with the ribavirin prodrugs in CD-1 Swiss mice infected with JE (Nakayama strain) or VEE (Trinidad donkey strain). Therapeutic efficacy was not observed with either ribavirin or the initial prodrug in these two models under the conditions of assay. The JE virus challenge was administered by the intracranial route, while the VEE virus was administered i.p. These studies have been repeated with the Beijing strain of JE virus administered i.p. to C57B1/6 mice and using a chemoprophylaxis protocol as employed in the earlier studies at Ft. Detrick. Increases in the mean survival time of drug-treated animals were noted. Additional *in vivo* studies with new ribavirin prodrugs and the first selenazofurin prodrug are planned. Additional ribavirin prodrugs have been synthesized and the first selenazole prodrug has been prepared in sufficient quantities for antiviral evaluation.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 85-23, Revised 1985).

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I. INTRODUCTION

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been found to possess broad-spectrum antiviral activity both *in vitro* and *in vivo* (1-5). Studies conducted at Fort Detrick have clearly demonstrated that ribavirin is markedly effective against bunyaviruses (e.g., Rift Valley fever virus) and arenaviruses (e.g., Lassa fever virus, Pichinde virus, and Machupo virus), but that it has only minimal to no efficacy against the alphaviruses (e.g., Venezuelan equine encephalitis virus and Chikungunya virus) or flaviviruses (e.g., Japanese encephalitis virus and yellow fever virus) *in vivo*. The apparent inability of ribavirin to achieve effective antiviral concentrations in the brain and central nervous system significantly limits its usefulness against those viruses which cause primary encephalitis (6). Ribavirin also does not prevent the late encephalitic phase of the diseases caused by Rift Valley fever, Junin, and Machupo viruses (7). The principal reason for this lack of efficacy is the relative inability of the drug to cross the blood-brain barrier and to concentrate in the central nervous system.

A related compound (2-β-D-ribofuranosylselenazole-4-carboxamide; selenazole) has been synthesized by Srivastava and Robins (8) and has been shown to exhibit potent, broad-spectrum *in vivo* activity against selected DNA-containing and RNA-containing viruses (9), including many viruses of potential military importance. Selenazole appears to be significantly more potent than ribavirin against paramyxoviruses, reoviruses, togaviruses, bunyaviruses, arenaviruses, picornaviruses, rhabdoviruses, herpesviruses, and pox virus *in vitro*. It is extremely active against yellow fever virus and is a prime candidate for antiviral chemotherapy studies *in vivo*. Another compound, tiazofurin, has been found to exert *in vitro* activity against flaviviruses and Korean hemorrhagic fever (KHF) virus. Both selenazole and tiazofurin appear to be rapidly excreted *in vivo*, so that a prodrug form of these drugs would be desirable to develop.

When selenazole is used in combination with ribavirin *in vitro*, synergistic antiviral effects are observed against Venezuelan equine encephalitis (VEE) virus and Japanese encephalitis (JE) virus (10). Synergistic activity has been shown for tiazofurin in combination with ribavirin against yellow fever virus *in vitro*, but not for other viruses. These observations indicate that combination antiviral chemotherapy with these agents and ribavirin might be a useful approach to the treatment of flavivirus infections *in vivo*.

Since many of the target viruses of interest to the Army produce a lethal encephalitis in the host, we believe that efforts directed toward the brain-specific delivery of candidate antiviral drugs will be an important approach to improving the efficacy of such drugs against agents of military significance.

Our initial efforts were directed toward the synthesis and evaluation of ribavirin prodrugs. Based upon the brain-specific delivery of, for example, phenethylamine (11), dopamine (12), trifluorothymidine (13), and acyclovir (14), we expected prodrug esters of ribavirin to effectively cross the blood-brain barrier. Once in the brain, the dihydropyridine moiety would be expected to be oxidized to the pyridinium salt which would be retained. Cleavage of the ester enzymatically would then produce a sustained delivery of ribavirin in the brain. For any specific compound, the rates of the various reactions in the process must be favorable, but the success achieved in several systems thus far certainly gave credence to our proposed application of this redox delivery system approach to the sustained, site-specific administration of antiviral agents such as ribavirin.

The early results obtained with our initial ribavirin prodrug (see First Annual report, dated October 31, 1986), were encouraging in that the compound protected mice from a lethal challenge with JE virus when administered by the i.p. route.

During the second year, we concentrated on developing pharmacological data on the metabolism and disposition of our initial ribavirin prodrug in mice, on the synthesis of new ribavirin prodrugs that are cleaved either more rapidly or more slowly than the original prodrug that was synthesized during the first year of this contract, and on the development of our *in vivo* antiviral evaluation systems involving lethal JE virus and VEE virus infections in mice. The initial ribavirin prodrug was evaluated in these animal infection models and the results compared with those obtained earlier at Fort Detrick. While the initial results obtained at USAMRIID could not

be confirmed, useful information was obtained with regard to the importance to the virus strain, route of inoculation, and the test protocol for additional antiviral evaluations in our laboratories at Southern Research Institute.

During the present year, further efforts were directed at the synthesis of additional prodrugs of ribavirin and we successfully completed the synthesis of the original prodrug form of selenazole in quantities sufficient for preliminary antiviral evaluation. The Beijing strain of JE virus was obtained and passaged in C57Bl/6 mice numerous times until the stock virus preparations produced 100% mortality in inoculated animals. Large stocks of challenge virus were prepared and titered *in vivo*. Antiviral chemotherapy experiments were conducted to evaluate three compounds (SRI-6711, SRI-7222, and SRI-7223) in this model system. While no reduction in mortality was observed, there were increases in the mean survival time of drug-treated mice compared with controls. Results of these experiments indicated that either higher dose levels or prolonged therapy might be even more effective.

II. CHEMISTRY

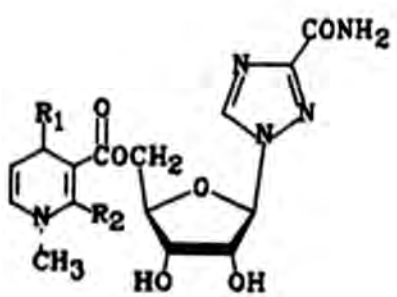
During the past year we have pursued four different areas. These are 1) the resynthesis of our original prodrug (5) by a new synthetic route, 2) the completion of the synthesis of a prodrug related to 5 but containing an extra methylene unit between the pyridine ring and the ester carbonyl (it turned out that we were forced to prepare the tetrahydro and not the dihydro compound), 3) the completion of the selenazofurin analog of the original prodrug, and 4) the synthesis of the original prodrug with either a 2-methyl or a 4-methyl group on the dihydropyridine ring, placed in an effort to slow down the hydrolysis of the prodrug. These two targets were selected based upon the results of the work reported in the last annual report on the radiolabelled 5.

Our current route to 5 is shown in Scheme 1, and is somewhat shortened from the one that we originally used. Conducting the coupling of the acid and the ester with triphenylphosphine and diethyl diazodicarboxylate eliminates the necessity of blocking the 2'- and 3'-hydroxyls with the isopropylidene group. The tetrahydro compound 11 is one that we reported on in the last annual report. Early this year we completed the preparation on a scale sufficient for the antiviral evaluation to be conducted. The route that was used is shown in Scheme 2. Compound 11 is one of a series that we proposed during the time that we expected that we would need to reduce the stability of the ester linkage toward cleavage. Our work with radiolabelled 5 told us that we were heading in the wrong direction, but we went ahead and prepared 11 because we already had worked out the synthesis. In the process we discovered that the series of compounds that we had chosen had major obstacles to overcome during their syntheses, and it was really better that we could propose and pursue other target structures.

We completed the synthesis of the original prodrug form (15) of selenazofurin on a scale sufficient for antiviral evaluation, using the routes shown in Schemes 3a and 3b. Our initial efforts toward 15 followed Scheme 3a, but after we had developed the route to 5 shown in Scheme 1, we applied this chemistry to selenazofurin as shown in Scheme 3b to simplify the route.

A major effort during the year was directed toward the synthesis of the prodrugs 18a and 18b. The route that we have pursued is shown in Scheme 4. Several unexpected problems occurred while we were following the steps shown in the scheme. First, in the step to 21 we found that methylation of a nitrogen in the triazole ring was a competing side reaction. Separation of the side product from the desired material proved to be quite difficult. Worse yet, the final reduction to produce 18 did not proceed with sodium dithionite as the reagent. We have explored a number of alternatives for this reduction, mentioned in the quarterly reports, and none of them has allowed formation of the desired compounds. Syntheses of the precursor methylated nicotinic acid derivatives are shown in Scheme 5.

During the year, new compounds submitted were 11 and 15, and additional amounts of 5 was also prepared. Considerable research was conducted on several other potential prodrugs, as well, as noted above. Experimental procedures for compounds and intermediates prepared during the year where those procedures are not given in a previous annual report are presented in the Experimental Section.

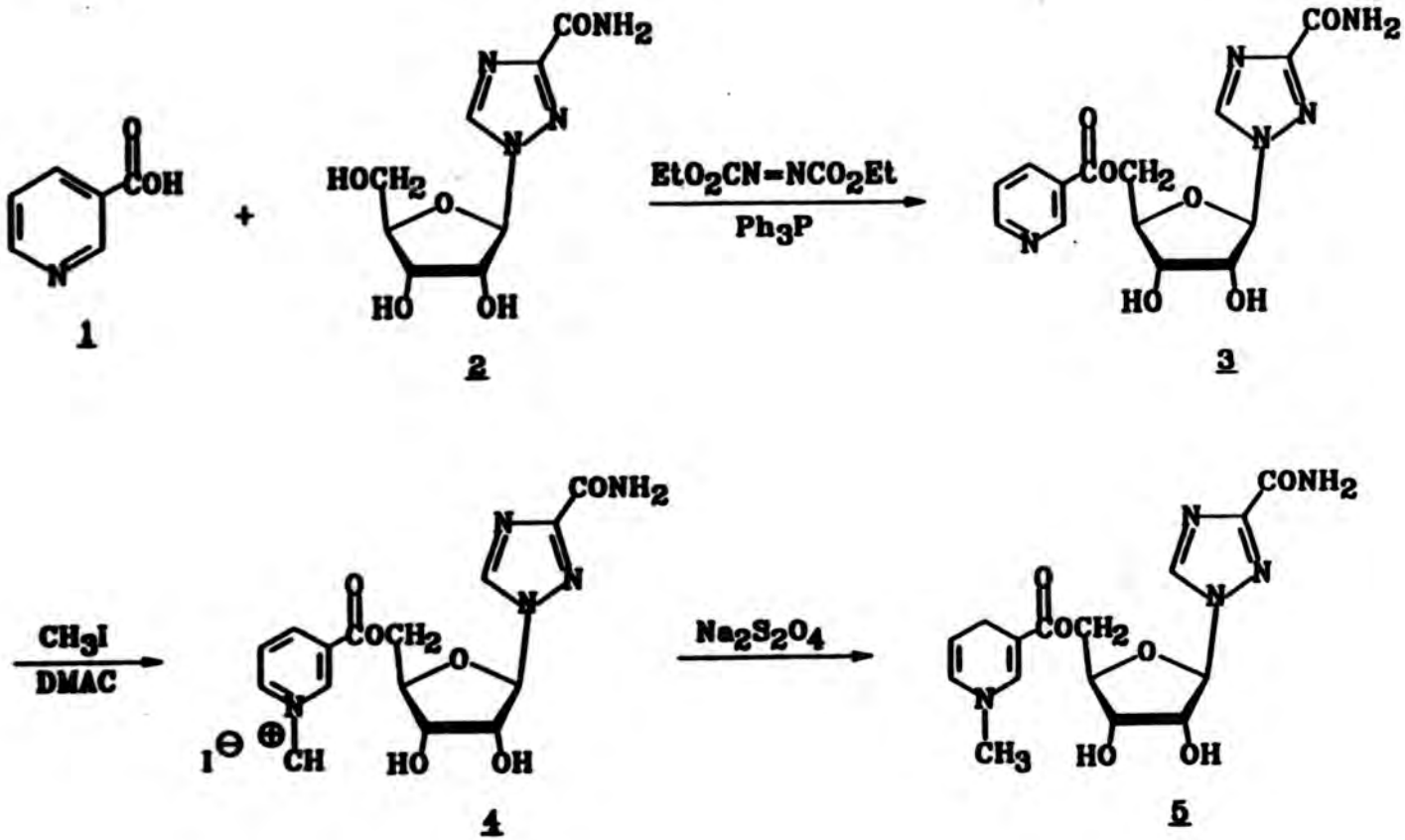


18

a) $R_1 = CH_3$, $R_2 = H$

b) $R_1 = H$, $R_2 = CH_3$

Scheme 1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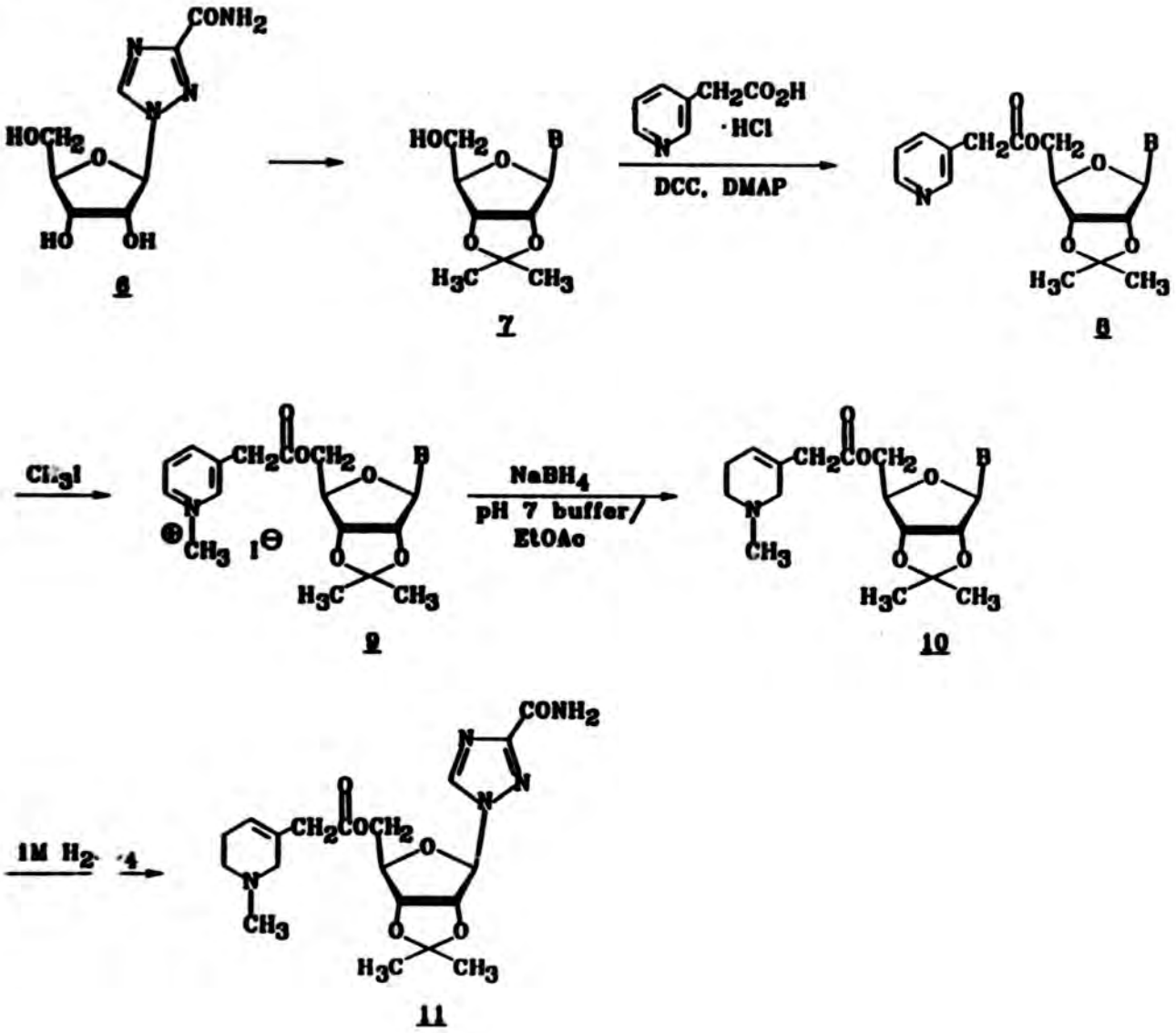
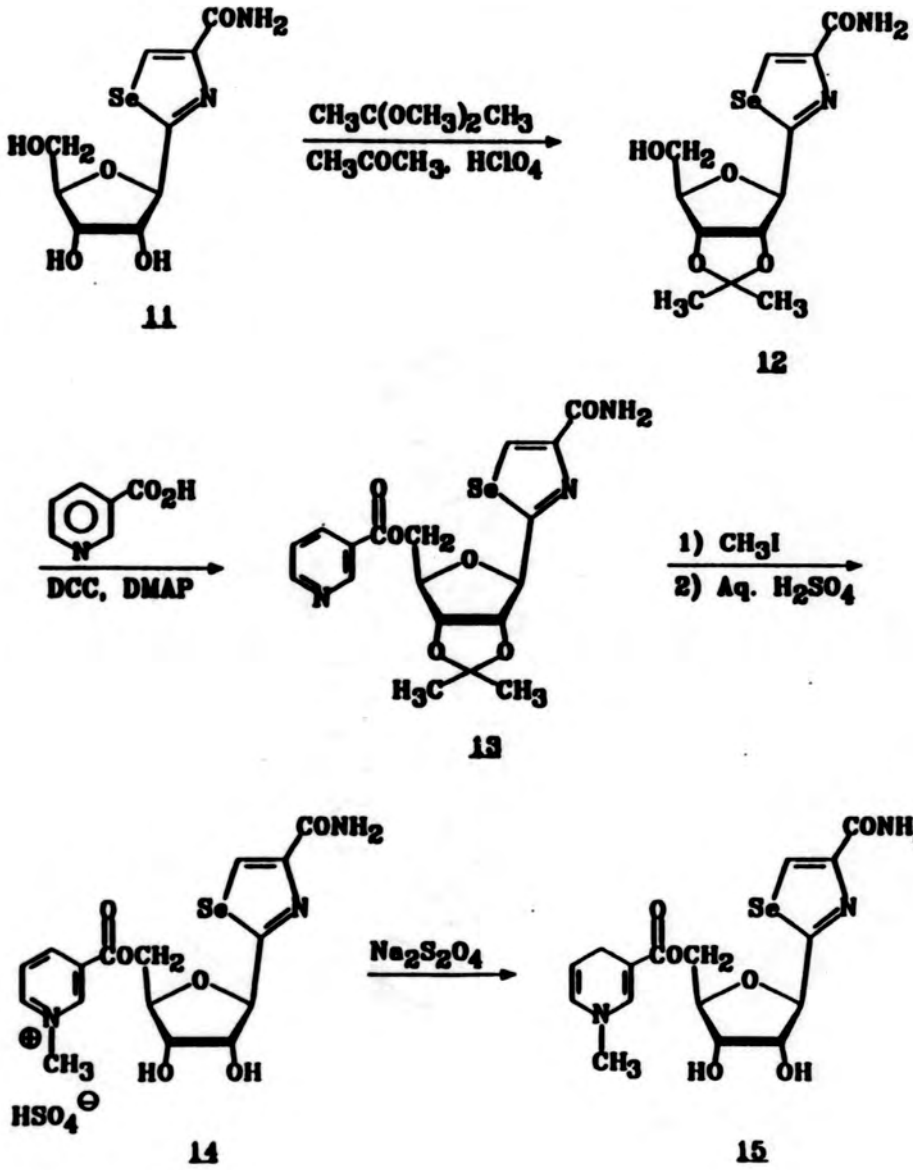
+
CONH2
HOCH2
HO
OH
2
EtO2CN=NCO2Et
Ph3P
COCH2
3
CH3I
DMAC
I⊖
⊕
CH
4
Na2S2O4
CH3
5

Scheme 2

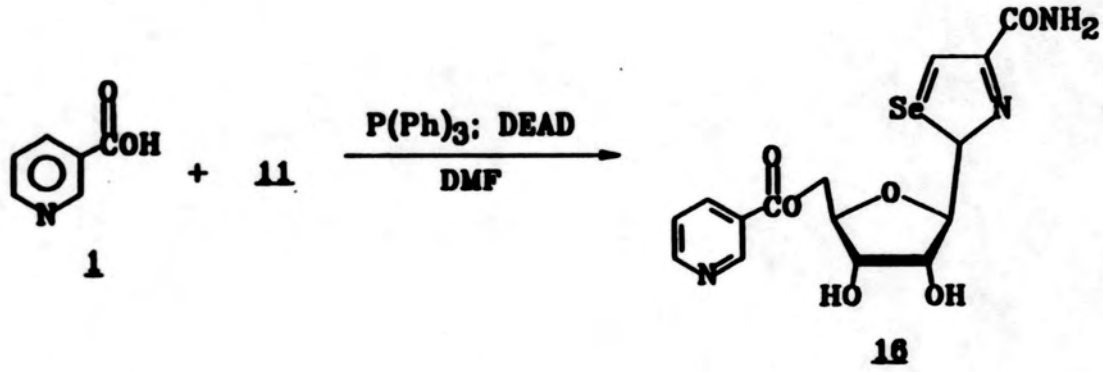


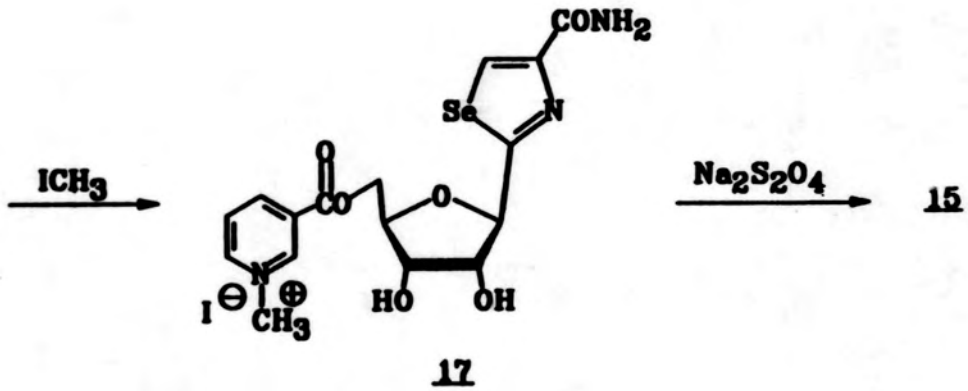
Scheme 3a



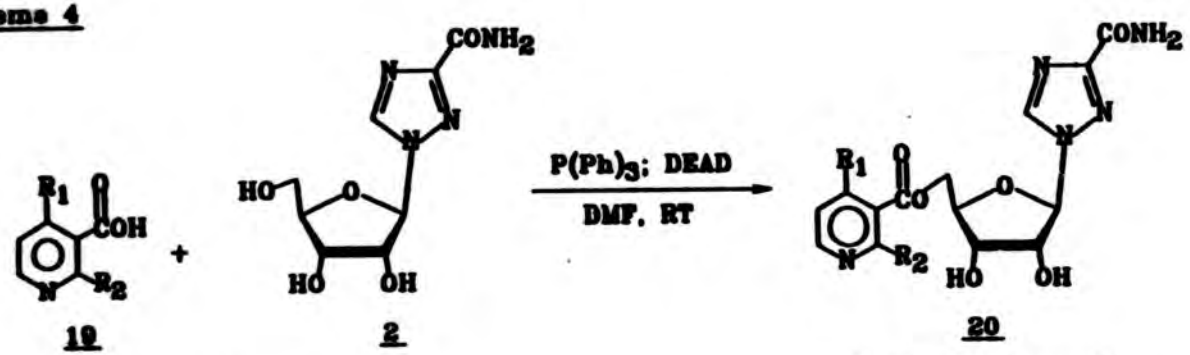
11 12 13 14 15

Scheme 3b



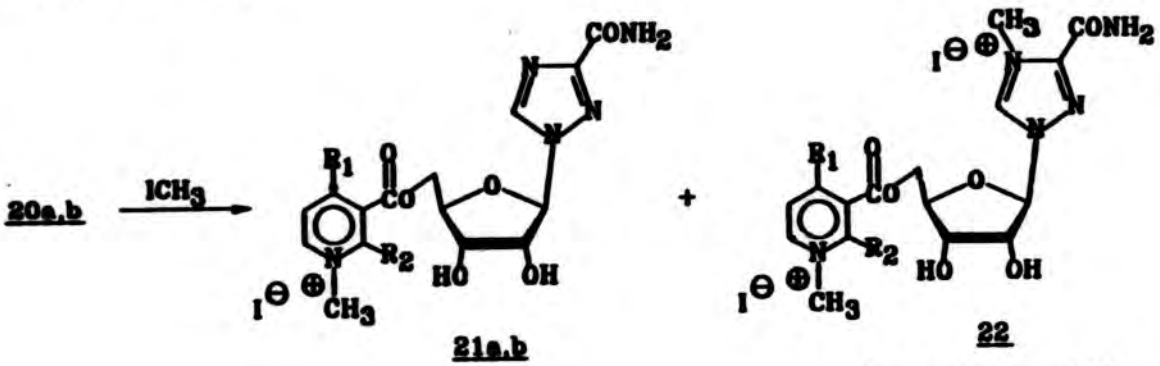


Scheme 4



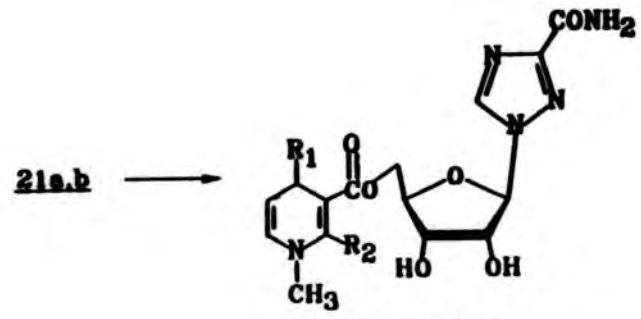
19 + 2 → 20

a) R_1 = H; R_2 = CH_3
b) R_1 = CH_3; R_2 = H



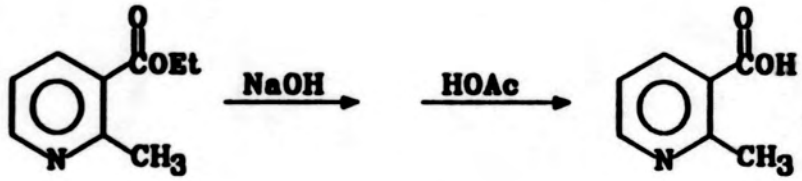
21a,b + 22

22: R_1 = H; R_2 = CH_3

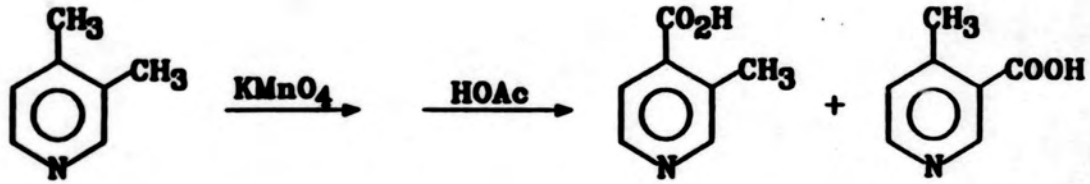


18a,b

Scheme 5



23 **19a**



24 **19b** **25**

A. Experimental Section

2-Methylnicotinic Acid (19a). Ethyl 2-methyl nicotinate (23, 10.33 g, 0.063 mol) in 100 mL of methanol was heated with NaOH (2.99 g, 0.065 mol) in 10 mL of H_2O for 2 h at 60-70 °C. The solution was adjusted to pH 5 with HOAc/H_2O and then concentrated to 10 mL. The solid was collected by filtration. The filtrate was concentrated and a second crop of crystals was collected. Total yield 7.42 g (0.054 mol; 86%). EIMS 137 (M); 119 (M - H_2O)$^+$. Mp 220 °C.

5'-O-(3-Pyridinylcarbonyl)ribavirin (3). To a solution of nicotinic acid (1, 93 mg, 0.5 mmol), ribavirin (2, 122 mg, 0.5 mmol) and triphenylphosphine (197 mg, 0.75 mmol) in 5 mL of DMF was added DEAD (118 ml, 0.75 mmol). After 4 h an additional equivalent each of triphenylphosphine and DEAD was added. The solution was then stirred at room temperature overnight. The solvent was evaporated (1 torr; room temperature) and the residue dissolved in a minimal amount of hot ethanol, from which the product crystallized. The product was collected and washed with EtOH. The filtrate was concentrated and a second crop was obtained identical to the first to give a total of 133 mg (0.32 mol; 64%) homogeneous by TLC (C/M 3:1). Mp 180-182 °C. FABMS: 350 (M + 1)$^+$. ^{1}H NMR (Me_2SO-d_6) d 9.08 (s, 1, H-2''), 8.88 (s, 1, H-5), 8.78 (d, 1, H-6''), 8.35 (d, 1, H-4''), 7.85 and 7.68 (2 s, 2, -C=O-NH_2), 7.55 (m, 1, H-5''), 5.95 (d, 1, H-1'), 5.74 (d, 1, HO-2'), 5.45 (d, 1, HO-3'), 4.60-4.35 (m, 4, H-2', H-3', and H-5'), 4.26 (s, 1, H-4'). HPLC (254 nm) 99%.

The reaction was repeated on a 20.5 mmol scale to yield 3.31 g (8.1 mmol; 40%) of material with similar analytical data.

5'-O-(1-Methyl-3-pyridiniumcarbonyl)ribavirin (4). A solution of 3 (1.11 g, 2.70 mmol) and iodomethane (0.4 mL, 3.2 mmol) in 15 mL of DMAC was stirred for 16 h, at which time 0.15 mL more ICH_3 was added and stirring continued at room temperature for 24 h. The product was precipitated with ether, collected and rinsed with ether to give 1.46 g (2.64 mmol; 93%) of a hygroscopic yellow solid. FABMS 364 M$^+$ of cation. ^{1}H NMR (Me_2SO-d_6) d 9.50 (s, 1, H-2''), 9.22 (d, 1, H-6''), 9.05 (d, 1, H-4''), 8.88 (s, 1, H-5), 8.28 (m, 1, H-5''), 7.90 and 7.65 (2 s, 2, -C=O-NH_2), 5.95 (d, 1, H-1'), 5.72 (d, 1, HO-2'), 5.45 (d, 1, HO-3'), 4.72 (dd, 1, H-5'a), 4.55 (m, 2, H-5'b and H-3'), 4.48 (m, 1, H-2'), 4.46 (s, 3, NH^+-CH_3), 4.32 (m, 1, H-4').

5'-O-(1-Methyl-1,4-dihydropyridinyl-3-carbonyl)ribavirin (5). To an ice-cold solution of 4 (1.45 g, 2.95 mmol) and sodium bicarbonate (1.5 g, 17.9 mmol) in 21 mL of degassed H_2O was added sodium dithionite (1.8 g, 8.8 mmol) under N_2. After 20 min the yellow precipitate was collected under N_2 and washed with ice-cold, degassed H_2O. The precipitate was then rinsed with anhydrous ether and dried (1 torr, room temperature) to give 497 mg (1.36 mmol; 46%). Thus, we have on hand ≈475 mg of prodrug suitable for use. FABMS 366 (M + 1)$^+$, 354 sugar$^+$, small amount of 245 (M + 1)$^+$ of ribavirin. HPLC analysis 87%. ^{1}H NMR pending. This material will be used without further purification.

4-Methylnicotinic Acid (19b).[1] To a solution of 3,4-lutidine (2.14 g, 0.02 mol) in 20 mL of H_2O was added $KMnO_4$ (3.90 g) in six equal portions over a period of three days, followed by 2.42 grams more on day 4. After a total of seven days Celite was added to the suspension and the suspension filtered over a Celite plug. The clear solution was then extracted with 3 x 100 mL of ether to remove the unreacted lutidine. The pH of the aqueous layer was then adjusted to 5 with glacial acetic acid and the solvent evaporated (20 torr; 60 °C). Attempts to selectively recrystallize the product were unsatisfactory. Thus, the crude product was dissolved in hot MeOH and absorbed onto silica gel and purified by flash chromatography ($CHCl_3$/MeOH/HOAc; 95:5:5) to give 492 mg (3.59 mmol; 18%) of **19b** and 225 mg (1.64 mmol; 8.2%) of **25**. Assignments were made by ^{1}H NMR (Me_2SO-d_6) d 2.56 (s, -CH_3) = **19b**; d 2.48 (s, -CH_3) = **25**.

The reaction was scaled up ten-fold and the products currently are being isolated presently.

Reference

1. K. Clarke, J. Goulding, R. M. Scrowston, *J. Chem. Soc.*, **1984**, 1501-05.

III. VIROLOGY

The Beijing strain of JE virus which was received and passed *in vitro* through Vero cell cultures has been used in all *in vivo* studies conducted during this reporting period.

A. JE Virus (Beijing Strain) Passage 2 Infection

1. Methods

In an effort to achieve 100% mortality, challenge levels of 5000, 2500, 1000, 500, 250, and 100 PFU of the passage 2 JE virus were inoculated into 4 week old CD-1 female mice. Virus challenged mice underwent saline treatment on a qld x 7 day schedule with the first saline dose administered day -1 to simulate compound treatment.

2. Results and Conclusions

As shown in Table 1, mortality rates in excess of 80% were not achieved at any challenge level. Maximum mortality occurred at a challenge level of 1000 PFU. The average day of death (ADD) ranged from 13 to 18 days post virus challenge. The results of this experiment diverge from those of the first challenge experiment conducted with this virus preparation. In the first experiment the mortality levels were higher than those that occurred in this experiment at comparable challenge levels. This difference may be due to dilution differences in the preparation of the two challenge inocula, differences in the storage and recovery of the virus in various cryovials or due to the outbred nature of the host mice.

Table 1
Mortality Rates of CD-1 Mice Bearing JE Virus
(Beijing Strain), Passage 2 Infection

Challenge Dose[1]	No. dead/No. infected	ADD[2]
5000 PFU	6/10	31.2 q 3.4
2500 PFU	5/10	13.8 q 4.4
1000 PFU	8/10	13.0 q 3.2
500 PFU	7/10	14.0 q 1.8
250 PFU	6/10	14.7 q 3.3
100 PFU	1/10	18.0 q 0.0

[1]administered intraperitoneally
[2]average day of death q 1 standard deviation

B. *In Vivo* Serial Passage of JE Virus

1. Methods

To enhance the virulence of the JE virus, it was serially passaged through CD-1 mice. Four week old female CD-1 mice were infected intraperitoneally with 1000 PFU of passage 2 JE virus. When the animals showed signs of sickness, they were sacrificed and the brains were prepared as a 10% homogenate in phosphate-buffered saline (PBS). A 1:100 dilution of this homogenate was used for intraperitoneal infection of a second group of 4 week old CD-1 female mice. The brains of sick animals were collected, prepared as a 10% homogenate and passed intraperitoneally into a third group of mice. Brain homogenates were prepared from these challenge animals and passed into a fourth group of animals.

2. Results and Conclusions

No increase in mortality was noted in the CD-1 mice after serial *in vivo* passage of the virus. Producing a JE challenge model in outbred CD-1 female mice by serial *in vivo* passage was not a viable alternative to the use of C57Bl/6 inbred strain mice.

C. *In Vivo* Challenge of 4 Week Old C57Bl/6 Mice

1. Methods

The lethal effects of intraperitoneally administered passage 2 JE virus were assessed in 4 week old C57Bl/6 female mice at challenge levels of 1000, 500, 250, 100 and 50 PFU per mouse.

2. Results and Conclusions

As shown in Table 2, 100% mortality was not achieved at any challenge level tested. At a challenge level of 1000 PFU, 66% mortality was achieved. The average day of death ranged from 12.7 to 15 days post virus infection. The virus preparation did not achieve 100% mortality at the dose levels tested either because the virus preparation is not as virulent as necessary or because the challenge doses were not high enough to induce 100% mortality.

Table 2
Mortality Rates of C57Bl/6 Mice Bearing JE Virus
(Beijing Strain), Passage 2 Infection

Challenge Dose[1]	No. dead/No. infected	ADD[2]
1000 PFU	6/9	12.7 q 2.3
500 PFU	5/10	12.8 q 2.5
250 PFU	4/10	15.0 q 4.2
100 PFU	2/10	13.0 q 1.4
50 PFU	0/10	NA[3]

[1]administered intraperitoneally
[2]average day of death q 1 standard deviation
[3]not applicable

D. Primary *In Vivo* Passage of *In Vitro* Virus Stock

1. Methods

Four-week-old female C57Bl/6 mice (Charles River Labs, Inc.) were challenged intracranially with JE virus (Beijing strain) to generate an initial *in vivo* passage pool. The virus used for challenge was the first Vero cell passage of JE virus which had been passaged in the C6/36 mosquito cell line (obtained from USAMRIID). Animals were administered one of three challenge levels (7800 pfu, 3900 pfu or 780 pfu) intracranially and sacrificed when they became moribund. The brains were harvested from the sacrificed animals and from those which were found shortly after death. The brains were prepared as a 10% homogenate in phosphate-buffered saline (PBS).

2. Results and Conclusions

The results of this experiment are shown in Table 3. Brain homogenates prepared from the animals challenged with 780 pfu will be subsequently referred to as Challenge Prep #1.

Table 3
Mortality Rates of C57Bl/6 Mice After
IC Administered JE Virus

	No. infected	No. dead	No. sacrificed
7800 pfu	5	2	2
3900 pfu	10	4	4
780 pfu	18	2	14*

*challenge Prep #1

E. Primary *In Vivo* Passage in C57Bl/6 Mice of *In Vivo* Virus Stock

1. Methods

A 10% brain homogenate generated from passage of JE virus (Beijing strain) in Swiss outbred mice (CD-1^R) was used as the challenge virus in this experiment. [The brain preps were prepared from CNS tissues collected from moribund CD-1^R mice infected intraperitoneally with the Vero cell passage 2 stock of JE virus]. Four-week-old female C57Bl/6 mice were challenged intraperitoneally or intracranially with one of several dilutions of the CD-1^R brain preps. Brains were collected from C57Bl/6 mice when they were moribund or recently dead. A 10% homogenate of the brains was prepared in PBS.

2. Results and Conclusions

The results of this experiment are shown in Table 4. The brain homogenates prepared from animals receiving a 1:100 dilution of the CD-1^R brain homogenate prep 1 will be subsequently referred to as Challenge Prep #2 while brain homogenates prepared from animals receiving a 1:1000 dilution of the CD-1^R brain homogenate prep 1 will be referred to as Challenge Prep #3.

Table 4
Primary *In Vivo* Passage in C57Bl/6 Mice of
In Vivo Virus Stock

Challenge	Dilution	Route	No. Infected	No. Dead	No. Sacrificed
CD-1^R prep 1	1:10000	IP	10	2	0
	1:1000	IP	10	3	6[a]
	1:100	IP	10	2	7[b]
CD-1^R prep 2	1:10000	IP	10	1	4
	1:1000	IP	10	4	6
	1:100	IP	10	3	7
CD-1^R prep 1	1:10000	IC	10	0	0
	1:1000	IC	10	0	6
	1:100	IC	10	1	8
CD-1^R prep 2	1:10000	IC	10	1	3
	1:1000	IC	10	1	9
	1:100	IC	10	1	9

[a]challenge Prep #3

[b]challenge Prep #2

F. Secondary *In Vivo* Passage

1. Methods

The brain preps generated in the primary *in vivo* C57Bl/6 mouse passages were inoculated into four-week-old C57Bl/6 female mice by the intraperitoneal or intracranial routes. Brains were collected from these animals when they were moribund or recently dead. The brains were prepared as 10% homogenates in PBS for use in challenge studies.

2. Results and Conclusions

The results of this experiment are shown in Table 5. The 10% homogenate prepared from the animals receiving the 1:100 dailution of Challenge Prep #1 are subsequently referred to as Challenge Prep #4. Challenge Prep #5 refers to the 10% homogenate prepared from animals receiving a 1:750 dilution of Challenge Prep #3.

Table 5
Secondary *In Vivo* Passage (IC and IP Routes)

Challenge	Dilution	Route	No. Infected	No. Dead	No. Sacrificed
Prep #1					
	1:100	IP	5	2	2[a]
	1:500	IP	5	5	0
	1:750	IP	5	1	3
	1:1000	IP	5	5	0
	1:100	IC	5	1	4
	1:500	IC	5		5
	1:1000	IC	5	1	4
Prep #2					
	1:100	IP	5	1	3
	1:500	IP	5	4	0
	1:750	IP	5	5	0
	1:1000	IP	5	4	1
	1:500	IC	5	4	1
	1:1000	IC	5	4	1
Prep #3					
	1:100	IP	5	3	2
	1:500	IP	5	4	0
	1:750	IP	5	1	3[b]
	1:1000	IP	5	3	0
	1:500	IC	5	2	3
	1:1000	IC	5	5	0

[a]challenge prep #4

[b]challenge prep #5

G. Challenge Experiment

1. Methods

The brain homogenates generated in the preceeding passage experiments were tested for lethality in four-week-old female C57Bl/6 mice. Mice were challenged by the intraperitoneal or intracranial route with various dilutions of the brain homogenates. Intraperitoneal challenge consisted of 0.5 ml of the inoculum while intracranial challenge consisted of 0.03 ml of the inoculum.

2. Results and Conclusions

With few exceptions, all of the preparations assayed produced 100% mortality in the challenged mice by the intraperitoneal and intracranial routes. This experiment indicates that JE virus capable of producing significant mortality in C57Bl/6 mice was generated by serial *in vivo* passage. The results are shown in Table 6.

Table 6
Mortality Rates of C57Bl/6 Mice Challenged by IC and IP Routes with Various Dilutions of Brain Homogenates

Challenge	Dilution	Route	No. Dead / No. Infected	ADD ± SD[a]
Prep #1				
	1:100	IP	4/5	14.3 ± 2.1
	1:500	IP	4/5	13.3 ± 4.0
	1:750	IP	5/5	12.6 ± 2.1
	1:1000	IP	5/5	14.0 ± 1.0
	1:100	IC	5/5	6.0 ± 0.7
	1:500	IC	5/5	6.0 ± 0.0
	1:1000	IC	5/5	6.0 ± 0.7
Prep #2				
	1:100	IP	5/5	13.2 ± 2.4
	1:500	IP	5/5	14.8 ± 1.6
	1:750	IP	5/5	13.6 ± 2.8
	1:1000	IP	5/5	13.8 ± 3.3
	1:500	IC	5/5	7.2 ± 1.1
Prep #3				
	1:100	IP	5/5	13.4 ± 1.5
	1:500	IP	5/5	12.8 ± 0.4
	1:750	IP	4/5	15.5 ± 2.6
	1:1000	IP	5/5	13.8 ± 1.3
	1:500	IC	5/5	7.4 ± 0.5
	1:1000	IC	5/5	8.4 ± 1.7
Prep #4				
	1:100	IP	5/5	10.6 ± 4.3
	1:500	IP	5/5	12.2 ± 2.2
	1:750	IP	5/5	13.4 ± 1.7
	1:1000	IP	3/5	14.0 ± 1.0
	1:100	IC	5/5	6.4 ± 0.9
	1:500	IC	5/5	6.8 ± 0.4
	1:750	IC	5/5	7.0 ± 0.7
	1:1000	IC	5/5	7.0 ± 0.0
Prep #5				
	1:100	IP	5/5	14.0 ± 1.0
	1:500	IP	5/5	13.0 ± 2.6
	1:750	IP	5/5	13.8 ± 2.5
	1:1000	IP	5/5	15.0 ± 1.6
	1:100	IC	4/5	6.8 ± 1.0
	1:500	IC	5/5	7.0 ± 0.7
	1:750	IC	5/5	6.2 ± 1.9
	1:1000	IC	5/5	7.0 ± 0.7
	1:1000	IC	5/5	7.4 ± 1.1

[a] ADD ± SD = average day of death ± 1 standard deviation

H. Confirmatory Challenge Experiment

1. Methods

To confirm the lethality of Challenge Preps 4 and 5 a second *in vivo* experiment was conducted. Four-week-old female C57Bl/6 mice were inoculated with 0.5 ml of one of several serial dilutions of Challenge Prep 4 or 5 by the intraperitoneal route.

2. Results and Conclusions

Dilutions of the Challenge Preps as high as 1:8000 produced significant mortality. The results, shown in Table 7, indicate that an acceptable challenge virus preparation has been obtained for use in young C57Bl/6 mice.

Table 7
Confirmatory Challenge Experiment
(Lethality of Challenge Preps #4 and #5)

Challenge	Dilution	No. Dead / No. Infected	ADD ± SD[a]	GMTD[b]
Prep #4				
	1:100	10/10	15.0 ± 1.8	14.9
	1:500	10/10	12.8 ± 1.7	12.7
	1:1000	10/10	12.0 ± 2.2	11.8
	1:2000	9/10	11.7 ± 3.2	12.3
	1:4000	9/10	13.9 ± 1.2	14.9
	1:8000	9/10	12.7 ± 2.5	13.5
Prep #5				
	1:100	9/10	13.2 ± 2.8	14.0
	1:500	9/10	14.2 ± 2.2	15.1
	1:1000	9/10	14.3 ± 1.9	15.2
	1:2000	7/10	14.6 ± 1.1	17.7
	1:4000	8/10	12.1 ± 2.6	14.0
	1:8000	8/10	13.0 ± 2.4	15.0

[a] ADD ± SD = Average day of death ± 1 standard deviation

[b] GMTD = geometric mean time to death

I. Preparation of a Large Stock of Challenge Virus

1. Methods

A lethal challenge virus preparation was successfully generated during the last reporting period (Challenge Prep #4) by serial *in vivo* passage of Beijing strain JE virus. During this reporting period a large pool of challenge virus was produced by intraperitoneal injection of 60 C57Bl/6 female mice with JE Challenge Prep #1. Brains were collected from these animals when they were moribund or recently dead. The brains were prepared as a 10% homogenate in phosphate buffered saline (PBS, pH 7.2) for use in challenge studies.

2. Results and Conclusions

A total of 47 brains were collected and homogenized. This preparation is subsequently referred to as Challenge Prep #6.

J. *In Vivo* Titration of Challenge Virus Stocks

1. Methods

Challenge Preps #4 and #6 were assessed for lethality in four-week-old C57Bl/6 mice. Mice were challenged intraperitoneally with 0.5 ml of serial dilutions of the brain homogenates prepared in PBS.

2. Results and Discussions

Both preparations are able to produce 100% mortality in challenged mice; however, the titers are significantly different. As shown in Table 8, Challenge Prep #4 produced significant mortality at a dilution of 1:60,000. Challenge Prep #6 was associated with significant mortality only at dilutions of 1:5,000 or less. The theoretical LD_{90}, LD_{50} and LD_{10} for Challenge Prep #4 calculated by plotting on probit paper are 1:52,000, 1:70,000 and 1:95,000 dilutions, respectively. The theoretical LD_{90}, LD_{50} and LD_{10} for Challenge Prep #6 calculated by plotting on probit paper are 1:2,600, 1:13,000 and 1:60,000 dilutions, respectively. Since Challenge Prep #6 is significantly less lethal than Challenge Prep #4 a second attempt to generate a large pool of Prep #4-like material should be pursued. The *in vitro* plaque forming unit (PFU) titers for both preparations should be determined.

K. Antiviral Evaluation of SRI 6711, SRI 7222 and SRI 7223

1. Methods

Female C57Bl/6 mice (VAF+, Charles River Labs) weighing 14-16 gms were randomly divided into treatment groups consisting of 15 mice each. The mice were treated intraperitoneally once daily for 9 days with one of the following (1) 10% DMSO in water, (2) 75 mg/kg SRI 6711, (3) 60 mg/kg SRI 7222, (4) 50 mg/kg SRI 7223, (5) 50 mg/kg ribavirin, (6) 25 mg/kg ribavirin or (7) 35 mg/kg selenazole. Compound treatments began the day preceding virus challenge. Compounds were administered two hours prior to challenge on the day of virus inoculation. Compound were prepared immediately before use by dissolving in 1 part 90% DMSO and diluting with 9 parts sterile water. Five of the mice were challenged intraperitoneally with 0.5 ml of a 1:8000 dilution of JEV Challenge Prep #4 (approximately 9 LD_{50}). The remaining five mice were challenged intraperitoneally with 0.5 ml of a 1:6000 dilution of JEV Challenge Prep #4 (approximately 4.5 LD_{50}). Untreated, infected and untreated, uninfected animals were included as controls.

2. Results and Conclusions

As shown in Table 9, no reduction in mortality occurred in any treatment group although there was some prolongation of the average day of death (ADD) in animals receiving SRI 6711 and challenged with a 1:16,000 dilution of the virus prep. The ADD of the mice receiving 75 mg/kg SRI 6711 was 14.8 days while the diluent-treated animals had an ADD of 11.9 days ($p = .02$). Although there was no reduction in the mortality for any treatment group the prolongation in ADD for SRI 6711 treated animals receiving the lower virus challenge implies that this compound may be effective if higher doses are administered for a longer treatment schedule and if the virus challenge is not excessive.

Table 8
Titration of Challenge Virus Stocks
(Challenge Preps #4 and #6)

Dilution	No. Dead/ No. Infected	ADD ± SD
Challenge Prep #4		
1:10,000	9/10	11.8 ±3.6
1:20,000	9/10	12.3 ±2.6
1:40,000	10/10	14.3 ±1.8
1:60,000	8/10	14.1 ±2.9
1:80,000	3/10	11.7 ±3.1
1:100,000	0/10	NA
1:150,000	1/10	15.0 ±0.0
1:200,000	0/10	NA
1:250,000	1/10	14.0 ±0.0
1:300,000	0/10	NA
1:400,000	0/10	NA
1:500,000	2/10	13.0 ±1.4
Challenge Prep #6		
1:1,000	10/10	12.8 ±2.0
1:5,000	8/10	13.0 ±2.1
1:10,000	1/10	14.0 ±0.0
1:20,000	1/10	15.0 ±0.0
1:40,000	2/10	16.5 ±0.7
1:60,000	1/10	11.0 ±0.0
1:80,000	0/10	NA
1:100,000	0/10	NA
1:150,000	0/10	NA
1:200,000	0/10	NA
1:250,000	0/10	NA
1:300,000	0/10	NA
1:350,000	0/10	NA
1:400,000	0/10	NA
1:450,000	0/10	NA
1:500,000	0/10	NA

Table 9
Antiviral Evaluation of SRI 6711, SRI 7222 and SRI 7223

		1:8,000[a]	1:16,000[b]
	No. dead/ No. Uninfected	No. dead/ No. Challenged (ADD)[c]	No. dead/ No. Challenged (ADD)[c]
untreated	0/5	10/10 (11.6 ± 2.6)	10/10 (12.5 ± 1.3)
10% DMSO/water	0/5	9/10 (12.7 ± 1.8)	10/10 (11.9 ± 2.1)
75 mg/kg SRI 6711	0/5	5/5 (12 ± 1.2)	5/5 (14.8 ± 1.6)[d]
60 mg/kg SRI 7222	0/5	5/5 (11.6 ± 3.1)	5/5 (13.2 ± 1.8)
50 mg/kg SRI 7223	0/5	5/5 (11.6 ± 1.1)	5/5 (12.2 ± 0.4)
50 mg/kg ribavirin	0/5	5/5 (13.6 ± 2.4)	5/5 (12.0 ± 1.2)
25 mg/kg ribavirin	0/5	5/5 (12.4 ± 1.8)	5/5 (13.0 ± 2.5)
35 mg/kg AVS253	0/5	5/5 (11.8 ± 0.4)	5/5 (11.6 ± 0.9)
1:5000 dilution challenge virus		10/10 (12.6 ± 2.3)	
1:8000 dilution challenge virus		10/10 (11.7 ± 1.3)	
1:10,000 dilution challenge virus		9/10 (12.7 ± 1.0)	
1:16,000 dilution challenge virus		10/10 (12.6 ± 1.4)	
1:20,000 dilution challenge virus		10/10 (12.2 ± 2.0)	
1:25,000 dilution challenge virus		10/10 (12.9 ± 1.4)	
1:30,000 dilution challenge virus		10/10 (12.8 ± 1.0)	
1:32,000 dilution challenge virus		9/9 (13.3 ± 1.7)	
1:40,000 dilution challenge virus		10/10 (12.1 ± 2.9)	
1:64,000 dilution challenge virus		8/10 (12.3 ± 2.6)	

[a]challenged intraperitoneally with 0.5 ml of 1:8000 dilution of virus prep

[b]challenged intraperitoneally with 0.5 ml of 1:16,000 dilution of virus prep

[c]ADD average day of death ± 1 Standard Deviation

[d]Students t-test p = 0.02

COMPOUND TEST DATA SHEET

EXP # JE827 VIRUS JE ID50 Challenge Prep 84 DOSE 0.5ml VIRUS DILUENT PBS CHAL ROUTE IP

SRI 8711, 7222,7223 AVS DILUENT 10%DMSO/water ROUTE & SCHEDULE qid X 9, IP FIRST AVS DOSE day -1 DAY 0 2 hrs pre

INOC DATE 5/27/89 TIME 11:35 TIME INOC ENDED 12:35 INOC BY B.Tower

SUPPLIER CRN DATE RECEIVED 5/17/89 CHALLENGE ROOM # 150 ENTRY DATE 5/27/89

CS7B1/6 female

CS	TREATMENT	date 5/26	27	28	29	30	31	6/1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	MD
		day -1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	
1	untreated	81	85	83	84	84	82	83	84	84			85				90							93								0/5
2	10%DMSO/water IP	81	84	84	899	85	84	86	86	86			87				92							95								0/5
3	175mg/kg SRI 8711	82	85	85	87	87	86	87	87	86			89				93							95								0/5
4	160mg/kg SRI 7222	80	82	81	83	83	82	84	85	84			87				92							95								0/5
5	150mg/kg SRI 7223	81	85	83	83	83	82	83	85	85			87				92							96								0/5
6	150mg/kg ribavirin	78	80	79	79	80	79	80	81	81			84				87							92								0/5
7	125mg/kg ribavirin	79	82	82	81	82	80	82	83	81			84				86							90								0/5
8	135mg/kg AVS 253	77	79	78	77	78	77	79	80	80			84				88							89								0/5
9	1:8000 dil of JEV untreated	80	82	83	82	82	81	81	83	81			59	1	2	1	1															5/5
10	1:8000 dil of JEV untreated	75	78	77	77	76	77	77	76	45 2			39		1		2															5/5
11	1:16000 dil of JEV untreated	80	81	82	82	82	82	84	84	83			66		2	2	1															5/5
12	1:16000 dil of JEV untreated	77	80	80	79	79	78	80	81	79			50 1	1		2	1															5/5
13	1:8000 dil of JEV 10%DMSO/water IP	78	81	81	80	80	80	81	83	82			69			2	28 1	1						18								4/5
14	1:8000 dil of JEV 10%DMSO/water IP	77	80	81	80	80	80	80	81	78		1	48	1	1	1	1															5/5
15	1:16000 dil of JEV 10%DMSO/water IP	80	82	81	81	79	78	80	81	77		1	33 1		1	2																5/5
16	1:16000 dil of JEV 10%DMSO/water IP	80	84	83	82	83	81	83	84	82		1	52	1	1	1	11 1	1														5/5
17	1:8000 dil of JEV 175mg/kg SRI 8711	81	84	84	84	84	83	86	87	83			61	2	2		1															5/5
18	1:16000 dil of JEV 175mg/kg SRI 8711	81	84	84	84	84	83	85	86	86			73			1	22 2	1	1													5/5
19	1:8000 dil of JEV 160mg/kg SRI 7222	83	84	82	82	81	82	84	83	67 1			51	2			9 1	1														5/5
20	1:16000 dil of JEV 160mg/kg SRI 7222	85	87	88	87	85	84	86	87	85			67		3		11 1	1														5/5
21	1:8000 dil of JEV 150mg/kg SRI 7223	74	75	74	73	73	73	75	76	76		1	48 1	1	2	1																5/5
22	1:16000 dil of JEV 150mg/kg SRI 7223	78	81	80	81	80	79	80	82	81			65		4	1																5/5
23	1:8000 dil of JEV 150mg/kg ribavirin	80	82	82	80	81	81	83	82	81			64	2			29			2	1											5/5
24	1:16000 dil of JEV 150mg/kg ribavirin	78	82	80	79	81	80	81	82	80			52 1		2	2																5/5

Comments: day 0 treatment started 9am treatment ended 9:35

CR#	TREATMENT	day	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	
		date	5/26	27	28	29	30	31	6/1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	80
25	1:8000 dil of JEV	75	77	77	76	78	76	78	78	74				48																			
	25mg/kg ribavirin										*			1	1			1	2													5/5
26	1:8000 dil of JEV	75	78	77	77	77	77	78	80	78				51					13														
	25mg/kg ribavirin													1		1	2				1											5/5
27	1:8000 dil of JEV	74	77	79	78	77	76	77	78	77				62																			
	35mg/kg AVS 253														1	4																5/5
28	1:16000 dil of JEV	79	81	79	79	79	76	78	80	81				63																			
	35mg/kg AVS 253														3	1	1															5/5
29	1:5000 dil JEV IP														2	2		1														5/5
30	1:5000 dil JEV IP									1					1			22														5/5
31	1:8000 dil JEV IP										1				1	3																5/5
32	1:8000 dil JEV IP													1	1	3																5/5
33	1:10000 dil JEV IP														1	1	1	2														5/5
34	1:10000 dil JEV IP															2	2	18						19								4/5
35	1:16000 dil JEV IP														1	1	2	12		1												5/5
36	1:16000 dil JEV IP													1		1	2	1														5/5
37	1:20000 dil JEV IP													1	1	2	1															5/5
38	1:20000 dil JEV IP													1	1		2	11			1											5/5
39	1:25000 dil JEV IP															1	2	10	1		1											5/5
40	1:25000 dil JEV IP														1	3		1														5/5
41	1:30000 dil JEV IP														2		2	1														5/5
42	1:30000 dil JEV IP															4	1															5/5
43	1:32000 dil JEV IP															1	1	10	1													4/4
44	1:32000 dil JEV IP													1		1		18	1	2												5/5
45	1:40000 dil JEV IP													1	1		1	12	1		1											5/5
46	1:40000 dil JEV IP										2				1	1		9		1												5/5
47	1:64000 dil JEV IP									1		1		1	1			10			1											5/5
48	1:64000 dil JEV IP														1		1	29		1				20								3/5

Comments: 6/3 (day 7) Cge 25-one animal had paralyzed hind quarters. BT

SRI 8711, SRI 7222 and SRI 7223 must be made fresh daily before injection. The ribavirin and AVS 253 may be prepared for multi-day dosing as they are more stable than the SRI compounds.

Treatment Protocol: First compound dose given day -1 Day 0 dose given 2 hr pre-

L. *In Vivo* Titration of JE Virus Challenge Prep 6 Stock

During this reporting period a second *in vivo* titration of JE virus Challenge Prep 6 was performed. Four week old female C57B1/6 mice were challenged intraperitoneally with the Challenge Prep 6 JE virus stock. Serial dilutions of the virus stock were prepared in PBS and administered intraperitoneally in a volume of 0.5 ml per mouse. The results of this titration indicate that a 1:4000 dilution of the virus stock should result in 90% mortality in four week old C57B1/6 mice after intraperitoneal challenge with 0.5 ml of the virus inoculum. The results of the *in vivo* titration are shown in Table 10.

Table 10
Titration of Challenge Prep 6 JE Virus Stock

Challenge Dose (dilution)	No. Dead/ No. Infected	ADD ± SD[a]	GMTD[b]
1:1000	10/10	12.9 ± 1.3	12.8
1:2000	10/10	12.7 ± 1.6	12.6
1:4000	9/10	12.2 ± 1.5	13.2
1:6000	2/10	14.0 ± 2.8	24.3
1:8000	8/10	13.1 ± 1.4	15.2
1:10000	4/10	15.8 ± 1.0	22.2

a. ADD ± SD = Average Day of Death ± 1 Standard Deviation

$$\text{ADD} = \frac{\Sigma[(\text{day of death}) \times (\text{number dead that day})]}{\text{total number of dead}}$$

b. GMTD = Geometric Mean Time to Death

$$\text{GMTD} = \sqrt[x]{n_1 \, n_2 \, n_x}$$ where n = day of death

x = total number of animals

All survivors are calculated as dying on Day 28

M. Preparation of Challenge Prep 7 JE Virus Stock

Because of the lower mortality associated with Challenge Prep 6 compared to Challenge Prep 4, another *in vivo* JE virus stock was prepared. Thirty-five female C57B1/6 mice were infected intraperitoneally with 0.5 ml of a 1:8000 dilution of JE virus Challenge Prep 4. The animals were sacrificed when moribund and the brains were collected and stored at -70°C. A total of 31 brains were collected and were pooled and prepared as a 10% homogenate in PBS for use in future challenge studies. This preparation will be referred to as Challenge Prep 7.

N. Preliminary Titration of JE Virus Challenge Prep 7

A preliminary range-finding titration of Challenge Prep 7 was performed. The virus stock was diluted in PBS and administered intraperitoneally to female C57B1/6 mice in a volume of 0.5 ml per mouse. As shown in Table 11, the mortality associated with Challenge Prep 7 is not unlike that present in Challenge Prep 6. The 1:5000 dilution of virus stock administered intraperitoneally resulted in 100% mortality while a 1:10000 dilution resulted in 40% mortality. A 90% lethal dose should be attained by administration of 0.5 ml of Challenge Prep 7 diluted to a final dilution between 1:5000 and 1:10000. The virus stock will be titrated at dilutions between 1:5000 and 1:10000 in order to determine the acceptable dilution necessary to produce a 90% mortality rate.

Table 11
Preliminary *In Vivo* Titration of Challenge Prep 7

Challenge Dose (dilution)	No. Dead/ No. Infected	ADD ± SD[a]	GMTD[b]
1:1000	10/10	11.6 ± 2.6	11.3
1:5000	10/10	13.7 ± 1.5	13.6
1:10000	4/10	14.3 ± 1.0	21.4
1:15000	1/5	14.0 ± 0.0	24.4

a. ADD ± SD = Average Day of Death ± 1 Standard Deviation

$$\text{ADD} = \frac{\Sigma[(\text{day of death}) \times (\text{number dead that day})]}{\text{total number of dead}}$$

b. GMTD = Geometric Mean Time to Death

$$\text{GMTD} = \sqrt[x]{n_1 \cdot n_2 \cdot n_x}$$ where n = day of death

x = total number of animals

All survivors are calculated as dying on Day 28

IV. PLANS FOR NEXT REPORTING PERIOD

A. Chemistry

On the basis of our work as well as the work of others, we can establish the following points:

1) The parent prodrug, with an N-methyl-1,4-dihydronicotinoyl group attached at 0-5′, was the most stable and easiest to prepare of all of the ones that we made.

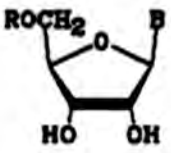
2) Insertion of one or more carbon between the 3-position of the dihydropyridine and the ester carbonyl reduces stability significantly and also makes it very difficult to prepare a 1,4-dihydro compound. These types of compounds appear unsatisfactory as prodrugs.

3) The parent prodrug is cleaved more rapidly than desired in the animals.

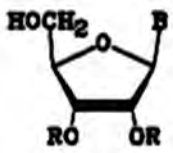
4) Preparation of prodrugs that have either a 2- or 4-methyl group on the dihydropyridine, designed to slow the cleavage of the ester in the prodrug, is also very difficult.

5) Attachment of the cucleoside through the group attached to the dihydropyridine is also very difficult.

Taking all of these facts into consideration, we propose to prepare more parent prodrug (1), as well as four other prodrugs (2-5). Attaching the standard dihydropyridine at either the 2′- or 3′-positions is expected to slow enzymic cleavage, and putting more than one group on the ribavirin nucleus may also have some desirable effects. The fifth prodrug, 5, is a new type that we hope will also be more slowly cleaved, but may also have the proper attributes for brain-targeting. All of these compounds will be examined in the animal model as they are prepared, and reevaluation will take place after each is examined.



1



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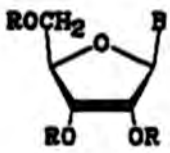

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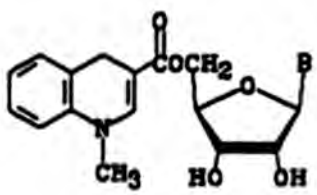

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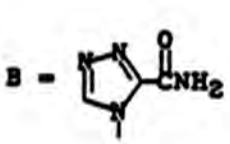

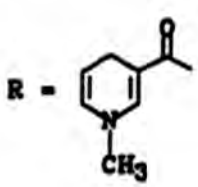

B. Virology

We will use several approaches to improve the efficacy of the ribavirin Bodor conjugate *in vivo*. Because the prodrug is rapidly cleaved, treatment schedules which will maintain higher levels of compound *in vivo* will be tested. First, more intensive dose schedules will be assessed. The conjugate will be administered on a q4hr schedule on Days 4 through 11 post-infection. If an increase in antiviral efficacy is seen with this treatment schedule, then constant drug infusion will be evaluated for efficacy. Infusions will be administered with 7 day pumps (ALZA Corp., Palo Alto, CA) implanted along the dorsal midline on Day 4 post infection.

In addition to altering the dosage schedule, we will increase the daily dose to the maximum tolerated in order to supply as much compound to the animals as possible. This in conjunction with decreasing the dosing interval should increase the available compound *in vivo*.

Information concerning dose schedules gathered from further evaluations of the original ribavirin conjugate will be used to determine the appropriate dosing regimen for new compounds which will be synthesized.

Preparation of a large pool of challenge stock with activity like challenge Prep #4 will be pursued further.

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