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DEVELOPMENT OF SYSTEMS FOR DELIVERY OF ANTIVIRAL DRUGS

ANNUAL REPORT

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This is our Fourth Annual Progress Report on Contract No. DAMD17-85-C-5276. It covers research performed during the period September 30, 1988 through September 14, 1989.

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 have been 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, Japanese encephalitis (JE) virus, and Punto Turo (PT) virus infections in mice. In preliminary studies at USAMRIID, 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. Initial attempts to confirm the original observation of *in vivo* efficacy of this ribavirin prodrug at Southern Research

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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 ribavirin prodrugs have been synthesized and evaluated in the JE model. While no reduction in mortality was observed, increases in the mean survival time of drug-treated animals were observed. Higher dose levels or prolonged therapy might be even more effective. An intracranial challenge model with Punta Toro virus was developed and target organ therapy protocols were designed for the evaluation of ribavirin and ribavirin prodrugs. Unfortunately, the contract period terminated before these latter models could be utilized for antiviral drug testing *in vivo*.

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) 86-23, 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 development of a prodrug form of these drugs would be desirable.

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 give 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 of the virus strain,

route of inoculation, and the test protocol for additional antiviral evaluations in our laboratories at Southern Research Institute.

During the third 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.

During the present year work was initiated on development of animal models relevant to the ongoing program. Two approaches have been used. First, an animal model which is sensitive to the antiviral effects of ribavirin has been pursued. This model is based on the differential lethality of the Balliet strain of Punta Toro (PT) virus. This virus is lethal when the challenge route is intracranal; while peripheral challenge is nonlethal. Thus, compounds with anit-PT activity would potentially be active if they crossed the blood-brain barrier.

Secondly, target organ systems are being developed such that small quantities of compound could be tested for *in vivo* efficacy prior to synthesis of bulk drug quantities. These systems are similar to those developed for herpes and vaccinia viruses (13,14). The virus inoculum is administered into the target organ. This is followed by administration of a single dose of compound into the target organ. Studies with VEE and JE are in progress to define the I.C. LD_{90} challenge levels. Following definition of the challenge dose, known *in vivo* active compounds will be tested for activity in the target organ system.

Scientific Progress During the Reporting Period

II. **Chemistry**

No chemistry work was done during this reporting period.

III. **Virology**

In order to better evaluate drugs designed to cross the blood brain barrier, especially ribavirin and selenazole derivatives, a new virus model is under development. This model involves the use of intracerebrally (i.c.) inoculated Punta Toro virus (strain Balliet), because of its sensitivity to ribavirin. This is being done because neither ribavirin nor the new derivatives synthesized at SRI which were designed to cross the blood brain barrier were active against intraperitoneally (i.p.) inoculated Venezuelan equine encephalomyelitis (VEE) virus. This data reflected the fact that ribavirin is minimally active against VEE *in vitro*.

The Balliet strain of PT was purchased from ATCC. A brain pool was prepared by passaging the virus in C57Bl/6 mice after intracerebral (i.c.) inoculation. An initial i.c. titration was performed (Table 1) and the LD_{90} appears to be at ≈ the 10^{-4} dilution. Additional titrations will be performed in which the mice are stressed by some type of treatment with PBS or Hank's balanced salt solution (HBSS). Another strategy is the development of a PT target organ evaluation system as previously developed by Allen, *et al.* (13,14) for herpes simplex and vaccinia viruses. In this type of model, the mice are inoculated i.c. with virus and then receive a single i.c. treatment with drug at an appropriate time post-virus inoculation. In order to evaluate the model and determine the best time for treatment, several groups of mice will be inoculated with PT and then different groups will receive the maximum i.c. tolerated dose of ribavirin at various intervals (4, 6, 8, 10 hours) after virus challenge. It is felt that pursuit of additional ribavirin derivatives for delivery across the blood brain barrier would be unwise if activity with ribavirin can't be achieved when it is put directly into the brain.

As another strategy to increase our *in vivo* potential for evaluation of drugs which might be effective against VEE or Japanese encephalitis (JE) viruses, we are developing target organ models for these viruses. An additional advantage of the target organ system is that it allows *in vivo* evaluation of drugs when only a small quantity of drug is available. We feel it would be feasible to evaluate compounds against VEE and JE which have shown activity *in vitro* and are available in approximately 120 mg quantities.

Results of intracerebral titrations with VEE and JE are given in Tables 2 - 4.

Table 1

Initial Intracerebral and Subcutaneous Titration of
Punta Toro (Balliet) in C57Bl/6 Mice

Group	No. Dead/ No. Challenged	ADD[a] ± 1 SD
$10^{-1.0}$ IC	10/10	6.2 ± 0.9
$10^{-1.5}$ IC	10/10	7.1 ± 0.9
$10^{-2.0}$ IC	10/10	7.7 ± 1.3
$10^{-2.5}$ IC	10/10	8.2 ± 1.0
$10^{-3.0}$ IC	10/10	8.7 ± 1.0
$10^{-3.5}$ IC	10/10	9.1 ± 0.9
$10^{-4.0}$ IC	8/10	10.1 ± 1.1
$10^{-5.0}$ IC	1/10	12.0 ± 0.0
$10^{-1.0}$ SC	0/10	N/A[b]
$10^{-2.0}$ SC	0/10	N/A
$10^{-3.0}$ SC	0/10	N/A
$10^{-4.0}$ SC	0/10	N/A
$10^{-5.0}$ SC	0/10	N/A
$10^{-6.0}$ SC	0/10	N/A

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

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

[b]N/A - Not applicable

Table 2

Initial Intracerebral Titration of VEE in Swiss Mice

Group	No. Dead/ No. Challenged	ADD[a] ± 1 SD
Uninfected Control	0/10	N/A[b]
Sham-infected	0/10	N/A
10^{6} pfu IC	10/10	5.9 ± 0.9
10^{7} pfu IC	10/10	6.5 ± 0.9
10^{8} pfu IC	5/10	6.8 ± 0.8
10^{9} pfu IC	2/10	7.0 ± 0.0

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

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

[b]N/A = Not applicable

Table 3

Second Intracerebral Titration of VEE in Swiss Mice

Group	No. Dead/ No. Challenged	ADD[a] ± 1 SD
Uninfected Control	0/10	N/A[b]
Sham-infected	0/10	N/A
$10^{7.1}$ pfu IC	10/10	6.1 ± 0.3
$10^{7.4}$ pfu IC	10/10	6.6 ± 1.2
$10^{7.7}$ pfu IC	8/10	7.0 ± 0.8
$10^{8.0}$ pfu IC	6/10	6.7 ± 0.8
$10^{8.3}$ pfu IC	8/10	6.4 ± 0.7
$10^{8.6}$ pfu IC	3/9	6.7 ± 0.6
$10^{8.9}$pfu IC	0/10	N/A

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

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

[b]N/A = Not applicable

Table 4

Initial Intracerebral Titration of JE in C57/Bl Mice

Group	No. Dead/ No. Challenged	ADD[a] ± 1 SD
Uninfected Control	0/5	N/A[b]
Sham-infected IC	0/10	N/A
1:10,000 IC	10/10	8.0 ± 1.4
1:50,000 IC	6/10	8.5 ± 2.8
1:80,000 IC	4/10	9.3 ± 2.1
1:100,000 IC	1/10	8.0 ± 0.0
1:200,000 IC	2/10	11.5 ± 2.1
1:400,000 IC	0/6	N/A
1:500,000 IC	1/10	11.0 ± 0.0
1:600,000 IC	0/10	N/A
1:800,000 IC	0/10	N/A
1:1,000,000 IC	0/10	N/A
1:2,000,000 IC	0/10	N/A
1:4,000,000 IC	0/10	N/A
1:6,000,000 IC	0/10	N/A
1:8,000,000 IC	0/10	N/A
1:10,000,000 IC	0/10	N/A

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

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

[b]N/A = Not applicable

C. **Plans for Next Reporting Period**

Since this project is terminating on March 14, 1989, we will write the draft final report to submit to SGD-RMI-S, Fort Detrick, Frederick, Maryland.

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