Dose Range-Finding Toxicity Study of RUC-4 Administered by Subcutaneous Injection in Mice

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ABSTRACT

RUC-4 is a novel αIIbβ3 antagonist being developed for prehospital therapy of myocardial infarction. The objective of this study was to determine the toxicity of RUC-4 when given as two subcutaneous (SC) injections separated by 4 hrs in C57BL/6 mice at 0, 10, 100, 300, and 1000 mg/kg/dose. At doses of 10 and 100 mg/kg, there were no test article-related effects on mortality, clinical signs, body weight, hematology, or bone marrow. Potential test article-related non-adverse clinical chemistry findings i-cluded increases in cholesterol on Day 2 (100 mg/kg) and decreases in triglyceride on Day 7 (10 and 100 mg/kg). Acute toxicity manifested as tremors, abnormal breathing, and/or lethargy that led to moribund removal or death shortly after the first dose (1000 mg/kg) or second (300 mg/kg) dose. Blood T_{max} of RUC-4 was observed 5-10 minutes after SC dosing. C_{max} and AUC_{last} values increased with increasing dose level, although the increases were not proportional to dose, suggesting non-linear kinetics. The apparent t½ of RUC-4 was 13.7-22.1 min and 31.7-45.3 min at the 10 mg/kg and 300 mg/kg dose levels, respectively. On Days 1 and 2, potential drug-related macroscopic lesions in RUC-4 dose groups included red discoloration (injection site, skin, urinary bladder wall) and gelatinous injection site. On Day 7, the only drug-related macroscopic lesion observed was red discoloration of the injection site in the 100 mg/kg group. On Days 1 and 2, drug-related microscopic lesions included subcutaneous hemorrhage and inflammation at various sites (all dose groups), skeletal muscle necrosis (injection site; abdominal, lumbar, head; 100 and 300 mg/kg), and coagulative necrosis of the renal tubules, hemorrhage of the muscularis and submucosa of the bladder, and acute inflammation of the bladder submucosa (300 mg/kg). On Day 7, microscopic lesions included subcutaneous hemorrhage and chronic inflammation at the injection site in females at 100 mg/kg. Based on these results, injection site, blood, urinary bladder, and kidney appear to be target organs of RUC-4 toxicity, with a Maximum Tolerated Dose of 100 mg/kg (mean AUC 870 min.µg/mL) and No Observed Adverse Effect Level (NOAEL) of 10 mg/kg/dose (mean AUC of 51 min.µg/mL) for BID dosing.

MATERIALS & METHODS

Test System:
- C57BL/6 mice (90/sex) received from Jackson Laboratory (Bar Harbor, ME).
- Approximately 8-9 weeks old and 16.3 g-25.6 g on Day 1.
- Certified rodent diet #2016C) and water (Birmingham public supply) available ad libitum.
- Female mice group housed (up to 5/cage) and male mice individually housed in solid bottom cages with hardwood bedding chips.
- Environmentally monitored, well-ventilated room. Temperature = 69.07-72.30° F. Relative humidity = 42.81%-71.72%. Lighting = fluorescent lighting approximately 12 hours/day
- Cage size and animal care conformed to guidelines of the U.S. Department of Agriculture (Animal Welfare Act; Public Law 99-198), to the *Guide for the Care and Use of Laboratory Animals*, and to applicable Standard Operating Procedures of Southern Research.

Test Article:
- RUC-4 Succinate received from DPI, NCAT, NIH (Rockville, MD) and stored frozen and protected from light and moisture.
- Vehicle, Physiological Saline Solution received from Butler Schein Animal Health (Dublin, OH).
- Dose formulations prepared by dissolving the appropriate amount of test article with vehicle under aseptic conditions followed by filtration through a 0.22-µm filter.
- Concentration analysis was performed on each formulation prior to and after completion of dosing on Day 1.

Group Assignment:
- Mice assigned to treatment groups by weight, using a computer-generated randomization procedure.
- Originally assigned to Groups 1-4 (Table 1), with 6 mice/sex/group for each core necropsy day.
- Due to excessive toxicity in the first few toxicokinetics animals dosed in Group 4, dosing in that group halted and remaining animals in the group (which had not been dosed), plus three additional mice/sex assigned to Group 5 (Table 1).

Table 1. Assignment of Animals to Dose Groups

Dose Group	Treatment	Dose Level (mg/kg/dose)	Total Dose (mg/kg/day)	Concentr. (mg/mL)	Number of Animals: Day 2 Core Group Necropsy	Day 7 Core Group Necropsy	Day 1 Toxicokinetics
1	Vehicle	0	0	0	6 M / 6 F	6 M / 6 F	3 M / 3 F
2	RUC-4	10	20	0.5	6 M / 6 F	6 M / 6 F	12 M / 12 F
3	RUC-4	100	200	5	6 M / 6 F	6 M / 6 F	12 M / 12 F
4	RUC-4	1000	2000	50	---	---	3 M / 3 F
5	RUC-4	300	600	15	6 M / 6 F	6 M / 6 F	12 M / 12 F

MATERIALS & METHODS, CONT'D

Treatment:
- RUC-4 Succinate administered by subcutaneous injection in the interscapular region.
- Dosing volume was 20 mL/kg body weight/dose (40 mL/kg/day for twice daily dosing).
- Mice in the Core Groups dosed twice (two separate injection sites) on Day 1 (~4 hours apart).
- Mice in the Toxicokinetics Groups dosed once on Day 1.

Observations:
- Mice observed twice daily for signs of mortality and moribundity.
- Core Group mice examined for clinical signs of toxicity ~30 minutes after each dose and at necropsy.
- All mice weighed during Week -1 (for randomization) and on Day 1 prior to dosing.
- Core Group animals weighed on Days 2 and 7 prior to necropsy.

Clinical Pathology:
- Blood samples for clinical pathology collected from Core Group animals on Days 2 and Day 7.
- On each day, 3 animals/sex/group used for hematology and 3 animals/sex/group used for clinical chemistry.

Blood Drug Levels:
- Blood samples for drug level determinations (BDLs) collected from TK animals on Day 1.
- Blood collected into Cryogenic tubes kept on ice containing 0.4 mL of 70:30 (v/v) deionized water:acetonitrile (HPLC/MS grade). Tubes capped and vortexed for 5-10 seconds at high speed, then separated into aliquots and stored frozen at approximately -70 °C.
- Animals assigned to subgroups as shown in Table 2, with three animals/sex in each subgroup.
- Each animal bled at two timepoints.

Table 2. Assignment of Animals to Subgroups for BDL Sample Collection

Subgroup	Timepoint 1 (Survival bleed)	Timepoint 2 (Terminal bleed)
Vehicle Control	----	10 minutes
1	Prior	40 minutes
2	5 minutes	80 minutes
3	10 minutes	2 hours
4 (Group 4)	20 minutes	--
5	20 minutes	4 hours

Sample and TK Analysis:
- Blood samples analyzed by solid phase extraction (SPE) followed by reverse liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).
- Blood drug level data were subjected to pharmacokinetic analysis using WinNonlin®; parameters calculated included Cmax, Tmax, AUC, and half-life.

Macroscopic and Microscopic Pathology:
- Days 2 and 7, animals in the Core Groups (6 animals/sex/group on each day) euthanized with complete postmortem examination.
- Tissues collected, fixed, and prepared for microscopic evaluation.
- Processed slides submitted to a veterinary pathologist for evaluation.

Dose Analysis:
- Nominal dose levels were 0, 10, 100, 300, and 1000 mg/kg/dose.
- Concentrations of RUC-4 Succinate in dose formulations corrected to convert mass of the succinate salt to mass of the free base (correction factor = 0.766) and for purity of the bulk chemical (98.11%).
- Corrected true dose levels are shown in Table 3.
- All results reported in terms of nominal dose levels.

Table 3. Nominal and Actual Dose Levels of RUC-4

Dose Group	Nominal Dose (mg/kg dose)	Actual Dose (mg/kg/dose)
1	0	0
2	10	7.52
3	100	75.2
4	1000	752
5	300	225.6

RESULTS

Mortality: A summary of mortality observed in this study is shown in Table 4. At doses of 10 and 100 mg/kg/dose (20 and 200 mg/kg/day on Day 1), there were no test article-related deaths. However, in the 1000 mg/kg dose group, all six animals (3/sex) from the TK Group that had been dosed either died or were euthanized as moribund within 1 hour after dosing on Day 1. One TK Group female was found dead prior to scheduled euthanasia on Day 1, but there were no deaths of male mice in the TK groups. Core Group animals in the 300 mg/kg/dose group survived long enough to receive their second dose, but these animals subsequently developed adverse clinical signs that were severe enough to cause death (4 males and 6 females) or warrant moribund removal (8 males and 6 females) of all animals on Day 1. There appeared to be a sex-related difference in sensitivity to RUC-4 Succinate in that the incidence of mortality was higher for female than for males. This sex-related difference was evident for TK animals (that received only one dose) and for Core Group animals (that received two doses).

Clinical Signs of Toxicity: A summary of clinical signs of toxicity observed in this study is shown in Table 4. There were no clinical signs in animals dosed at 0, 10, or 100 mg/kg/dose. Clinical signs observed in animals dosed at 300 mg/kg/dose included abnormal breathing, lethargy, tremors, and found dead. Clinical signs were not required or collected for the six TK animals in Group 4 that were dosed. For both sexes, the incidences of abnormal breathing, lethargy, and tremors were higher for animals that received two doses (Core Groups) than for those that received one dose (TK animals). There appeared to be a sex-related difference in sensitivity to RUC-4 Succinate in that female mice in the 300 mg/kg/dose group had higher incidences of abnormal breathing, lethargy, and found dead than males. This sex-related difference was evident for TK animals (that received only one dose) and for Core Group animals (that received two doses).

Table 4. Mortality and Clinical Signs of Toxicity Observed in Mice Treated with RUC-4 Succinate by Subcutaneous Injection

Dose Group	Dose Level (mg/kg/dose)	Mortality and Clinical Signs (Incidence); Males	Mortality and Clinical Signs (Incidence); Females
1	0	None (12/12)	None (12/12)
2	10	None (12/12)	None (12/12)
3	100	None (12/12)	None (12/12)
5, Core Received Two Doses	300	Tremors (12/12) Abnormal Breathing (9/12) Lethargy (8/12) Found Dead (4/12) Moribund Euthanasia (8/12)	Tremors (12/12) Abnormal Breathing (12/12) Lethargy (12/12) Found Dead (6/12) Moribund Euthanasia (6/12)
5, TK Received One Dose	300	Tremors (1/12) Abnormal Breathing (5/12) Lethargy (2/12)	Tremors (9/12) Abnormal Breathing (9/12) Lethargy (9/12) Found Dead (1/12)

Body Weights: There were no test article-related effects of RUC-4 Succinate on body weight at any dose level. For animals in the 300 mg/kg/dose group, this was due to the fact that animals died or were removed from study on the same day of dosing.

Clinical Pathology: There were no test article-related effects of RUC-4 Succinate on hematology parameters on in animals dosed at the 0, 10 and 100 mg/kg/dose levels. Potentially test article-related clinical chemistry findings were considered equivocal due to the low number of mice/group and parameter values that were comparable with literature values. These findings included statistically significant increases (+30.3%) in cholesterol levels on Day 2 in female mice at 100 mg/kg/dose and statistically significant decreases in triglyceride levels on Day 7 in male mice at 10 and 100 mg/kg/dose (-41.1% and –44.0% respectively). However, neither the increases in cholesterol levels nor the decreases in triglyceride levels were considered biologically relevant.

Toxicokinetics: Analysis of the BDL blood samples showed that some of the samples collected for the pre-dosing timepoint showed low levels of contamination with test article. However, the concentrations of RUC-4 in these samples were low enough to not have an effect on data interpretation.

Results of the toxicokinetic analysis of the BDL data are shown in Table 5. Toxicokinetic parameters were derived from noncompartmental analysis of mean blood concentrations of RUC-4 over time. No sex-related differences in toxicokinetic parameters were apparent. Peak concentrations of RUC-4 were observed in blood 5-10 minutes after SC administration. C_{max} and AUC_{last} values increased with increasing dose level; however, the increases in both parameters were greater than the increases in dose level. These non-linear increases were most evident for male and female animals in the 300 mg/kg/dose group where C_{max}/Dose and AUC_{last}/Dose ratios were 2- to 3-fold and 6- to 7-fold higher, respectively, than the corresponding values observed for animals in the 10 mg/kg/dose group. The apparent terminal elimination half-life of RUC-4 in blood appeared to increase with increasing dose level and was 13.7 (males) and 22.1 minutes (females) for mice in the 10 mg/kg/dose group and 45.3 (males) and 31.7 minutes (females) for mice in the 300 mg/kg/dose group.

RESULTS

Table 5. Toxicokinetic Parameters Calculated from Concentrations of RUC-4 in Blood following SC Administration to Mice

Group	Dose (mg/kg)	Sex	T_{max}[a] (min)	C_{max}[b] (ng/mL)	t½[c] (min)	AUC_{last}[d] (min·µg/mL)	AUC_{INF}[e] (min·µg/mL)
2	10	M	5	2570	13.7	58.9	59.9
2	10	F	5	1895	22.1	42.4	46.6
3	100	M	5	30700	NR	1058	NR
3	100	F	10	33867	NR	683	NR
5	300	M	10	139433	45.3	10305	10585
5	300	F	10	154667	31.7	9210	9290

NR: Not reported; terminal phase parameters are not reported due to the poor fit (R^2 adjusted < 0.8000) of the data to the terminal portion of the elimination curve
[a]Time mean maximum concentration of RUC-4 was observed in blood
[b]Mean maximum concentration of RUC-4 observed in blood
[c]Apparent half-life of the terminal phase of elimination
[d]Area under the blood concentration versus time curve calculated from 0 to the last time point the mean concentration of RUC-4 in blood was above the level of quantitation
[e]Area under the blood concentration versus time curve calculated from 0 to infinity

Macroscopic Pathology: For mice in the 300 mg/kg/dose group, drug-related macroscopic lesions included red discoloration of the injection site, abdominal skin, and urinary bladder, and skin. For mice that survived past Day 1, drug-related macroscopic lesions included red discoloration of the injection site (10 and 100 mg/kg/dose), red discoloration of the abdominal skin (10 and 100 mg/kg/dose), and red discoloration of the skin (100 mg/kg/dose) on Day 2; and red discoloration of the injection site (100 mg/kg/dose) on Day 7.

Microscopic Pathology: Microscopic lesions observed for mice in the 10 and 100 mg/kg/dose groups on Day 2 included subcutaneous hemorrhage, subcutaneous inflammation, and skeletal muscle necrosis. On Day 7, the only remaining microscopic lesions were in female mice and included minimal to mild subcutaneous hemorrhage and subcutaneous chronic inflammation.

CONCLUSIONS

- RUC-4 Succinate was well-tolerated when administered twice daily by subcutaneous injection at 10 or 100 mg/kg/dose (20 or 200 mg/kg/day). At these doses there was no mortality and no clinical signs of toxicity.
- RUC-4 Succinate was severely toxic when administered twice daily at 300 mg/kg/dose (600 mg/kg/day) and above. Clinical signs of toxicity included abnormal breathing, lethargy, and tremors. 100% of mice at this dose either died or were euthanized as moribund.
- RUC-4 was toxic at a single dose when administered once at a dose of 300 mg/kg. Clinical signs of toxicity were similar to those observed in animals dosed twice daily at the same dose, but only 1/24 animals died after a single dose.
- Female mice appeared to be more sensitive to toxicity from RUC-4 Succinate than were males.
- Possible test article-related changes in clinical chemistry findings included increases in cholesterol on Day 2 and decreases in triglyceride levels on Day 7.
- Hemorrhages observed on microscopic examination were consistent with the known inhibitory action of RUC-4 on platelet aggregation.
- RUC-4 Succinate-associated toxicity appeared to have resolved for the most part by Day 7 after in mice receiving doses of 10 or 100 mg/kg/dose.
- C_{max} and AUC_{last} values with increasing dose level, although these increases were not dose-proportional.
- RUC-4 exhibited non-linear kinetics at the dose levels administered, suggesting saturated elimination and/or metabolism of RUC-4.
- No sex-related difference in toxicokinetics was observed.

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