CENTER FOR DRUG EVALUATION AND RESEARCH

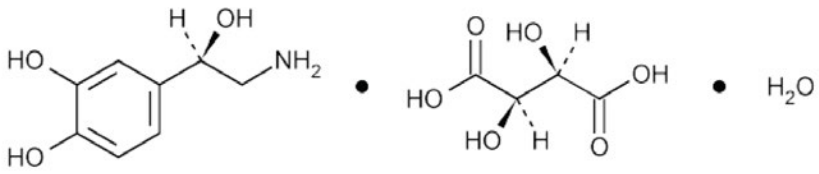
APPLICATION NUMBER:

214628Orig1s000

NON-CLINICAL REVIEW(S)

Memorandum

Date:	April 15, 2022
Application No:	NDA 214628
From:	Rama Dwivedi Pharmacologist Xuan Chi Supervisory Pharmacologist
Drug Product:	Norepinephrine Bitartrate, USP.
IUPC Name(s):	4-[(1R)-2-amino-1-hydroxyethyl]benzene-1,2-diol;(2R,3R)-2,3-dihydroxybutanedioic acid; hydrate



Molecular Formula:	$C_8H_{11}NO_3.\ C_4H_6O_6.\ H_2O$
Molecular Weight:	337.28
Indication:	Restoration of blood pressure in adult patients with acute hypotensive states
Subject:	Safety Evaluation of (b) (4) impurity (b) (4)
Applicant:	Nevakar, Inc. (Nevakar)
Reference Listed Drug:	Levophed™ (NDA 007513)

Background Information

Nevakar, Inc. (Nevakar) resubmitted the New Drug Application (NDA 214628) for Norepinephrine in Sodium Chloride Injection (16 mcg/mL, 32 mcg/mL, and 64 mcg/mL) ready-to-use product for intravenous administration on 4/8/2022, as per accordance with 505(b)(2) of the Federal Food, Drug and Cosmetic Act.

The nonclinical basis for this submission is Hospira's Levophed™ (NDA 007513; approved on July 13, 1950); the reference listed drug (RLD). The route of administration, indication, as well as the dosing regimen for Nevakar's drug product are the same as the listed drug (LD), Levophed™. The 14-day repeat-dose toxicity study aimed at qualifying the impurities (RRT (b) (4) and RRT (b) (4)) has been reviewed (see review in DARRTS dated 5/6/2021).

Nevakar is relying on Agency's prior findings of safety and efficacy for Levophed, the LD, to support the NDA. For the original NDA submission, Nevakar conducted searches of the literature to evaluate additional published toxicological information available since 26 September 2007 (the date of the LD label). To support the resubmission, Nevakar has now conducted nonclinical literature searches from 7th November 2020 till 15th January 2022. The most current approved LD label is dated 10/21/2020.

In the resubmission, during the safety assessment of Norepinephrine Bitratrate RTU Injection (NVK004) drug product, Nevakar identified an additional impurity (b) (4), also identified as impurity (b) (4). The sponsor proposed a specification of (b) (4)% for (b) (4) in the final drug product. According to ICH-Q3B, for a drug product with MDD of (b) (4) mg, the qualification threshold should be established at NMT 200 µg total daily intake. The proposed threshold would produce total daily intake of (b) (4) ug, which needs toxicological qualification or a reasonable justification.

To qualify this impurity for Norepinephrine Bitratrate RTU Injection (NVK004) drug product, Sponsor conducted the (Q)SAR analysis using CASE Ultra version 1.8.0.to assess the mutagenic potential. The sponsor also utilized the publicly available toxicology data on impurity (b) (4) that include Ames assay, in vitro mammalian cell chromosomal aberration assay, in vivo mammalian erythrocyte micronucleus test, unscheduled DNA synthesis (UDS) test with mammalian liver cells in vivo, 13-week repeated-dose oral and subcutaneous toxicity studies in rats and reproductive toxicology studies in rats.

Table 1: Summary of (Q)SAR Results

Name Structure CAS #	CASE Ultra Results		M7 Class
	GT1_BMUT 1.8.0.1.11479.500	GT1_EXPERT 1.8.0.1.11479.500	
(b) (4)			Class 5

* Calculated probability (Cp)

Based on the weight of evidence approach per ICH-M7(R1) and ICH-S2(R1), Impurity (b) (4) is considered non-genotoxic and a Class 5 impurity. This is based on (Q)SAR analysis (negative) and the results from the battery of genotoxicity testing (negative Ames assay, positive in vitro mammalian cell chromosomal aberration assay, and negative in two in vivo genotoxicity assays).

The Permissible Daily Exposure (PDE) calculated from the NOAEL ((b) (4) mg/kg/day) in the 13-week rat oral toxicity study is (b) (4) ug, as per ICH-Q3C/Q3D approach, using modification factors of 5, 10, 2, 10 to account for extrapolation between rat and human, variability between individuals, toxicity study of shorter duration and different route of administration, respectively. This provides a safety margin of (b) (4). Using the same approach, the PDE calculated from the NOAEL ((b) (4) mg/kg/day) reported in the 13-week rat sub-cutaneous toxicity provides only a (b) (4) safety margin.

Recommendation

Considering the endogenous nature of the impurity (b) (4), the acute use of the drug product (less than 10 days over a life time) for a life-threatening indication and the safety margin obtained from 13-weeks repeated dose toxicology studies, the proposed specification level of (b) (4)% for impurity (b) (4) in Norepinephrine Bitartrate Injection RTU- drug product is considered acceptable from the Pharm/Tox perspective.

REFERENCES

ICH M7(Rl). Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk (2017). https://www.ich.org/. Accessed February 2022.

(b) (4)

Schuster J, Fabri M, Eming S, et al. Allergic Drug Eruption Secondary to Intravenous Acyclovir. Acta Derm Venereal. 2008;88(2):196-8.

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/s/

RAMA S DWIVEDI
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DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration
Center for Drug Evaluation and Research Office of Drug Evaluation I
Division of Cardiovascular and Renal Products

Memorandum

Date: May 6, 2020

From: Rama Dwivedi
Pharmacologist

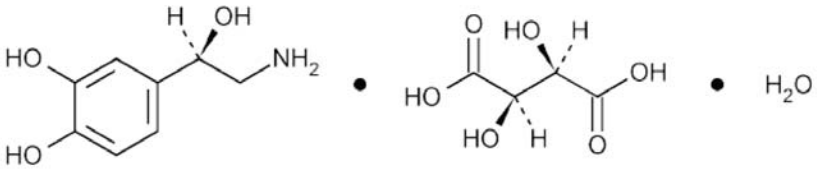
Xuan Chi
Team Leader

To:

Application No: NDA 214628

Drug Product: Norepinephrine Bitartrate, USP.

IUPC Name(s): 4-[(1R)-2-amino-1-hydroxyethyl]benzene-1,2-diol;(2R,3R)-2,3-dihydroxybutanedioic acid; hydrate



Molecular Formula: $C_8H_{11}NO_3 . C_4H_6O_6 . H_2O$
Molecular Weight: 337.28

Indication: Restoration of blood pressure in adult patients with acute hypotensive states

Subject: Safety Evaluation of two impurities (b) (4) (b) (4)

Applicant: Nevakar, Inc. (Nevakar)

Reference Listed Drug: Levophed™ (NDA 007513

Background Information

Nevakar, Inc. (Nevakar) submitted the New Drug Application (NDA 214628) for Norepinephrine in Sodium Chloride Injection (16 mcg/mL, 32 mcg/mL, and 64 mcg/mL) ready-to-use product for intravenous administration, as per accordance with 505(b)(2) of the Federal Food, Drug and Cosmetic Act.

The basis for this submission is Hospira's Levophed™ (NDA 007513; approved on July 13, 1950); the reference listed drug (RLD). The route of administration, indication, as well as the dosing regimen for Nevakar's drug product are the same as the listed drug (LD), Levophed™.

Nevakar conducted a search of the literature to evaluate additional published toxicological information available since 26 September 2007 (date of current LD label in April 2020); and, an updated LD label has since been approved (15 June 2020).

The formulation of Nevakar's drug product contains two impurities at RRTs of ~ (b) (4) and ~ (b) (4) found present in stability batches (Table 1). These two impurities are (b) (4)

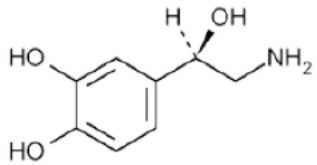
Table 1. Two impurities at RRTs of (b) (4) found to be present in stability batches

Related Substances (HPLC)	Related Compound (b) (4)	NMT (b) (4)%	NMT (b) (4)%	In-house by HPLC
	Related Compound	NMT %	NMT %	
	Any unspecified impurity	NMT %		
	Total impurities	NMT %	NMT %	

(b) (4)

Figure 1: Structures of Norepinephrine and RRT (b) (4) and RRT (b) (4) Impurities

Norepinephrine



RRT (b) (4) | RRT (b) (4)

(b) (4)

To support the limits for the two specified impurities, NMT (b) (4)% (RRT (b) (4)) and NMT (b) (4)% (RRT (b) (4)) that exceeded the qualification threshold of 0.5% or 200 mcg TDI, Nevakar conducted 28 day

toxicology studies (GLP 28-day continuous infusion rat study, GLP) Ames Test, *in vitro* Chromosomal Aberration Test, and *in vivo* Mouse Micronucleus test and in vitro hemolysis studies as below.

Table 3. Toxicology Studies conducted by Nevakar

Type of Study	Species (strain)	Method of Administration	GLP Compliance	Study Number
Other Studies				
Hemolytic Potential Assay	Rat, dog, monkey, and human blood	in vitro	No	16NEVAP1R1
Impurity Studies				
28-day Repeat-Dose Toxicity Study	Rat (Sprague Dawley)	Intravenous	Yes	1602-19287
Ames Test	*S. typhimurium* and *E. coli*	in vitro	Yes	52578
Chromosomal Aberration Test	Chinese Hamster V79 cell	in vitro	Yes	(b) (4) 20AA1089-2
Micronucleus Assay	Mouse (Swiss Albino)	in vivo	Yes	53153

Abbreviations: GLP=Good Laboratory Practices

The identification threshold for this unspecified impurity (NMT (b) (4)% each) is based on ICH guidance Q3B(R2) for drug products with a maximum daily dose (MDD) of (b) (4) mg.

Table. 2. Dose Volume Required for MDD of (b) (4) mg

NVK004 Concentration (mcg Norepinephrine/mL)	Dose Volume to achieve (b) (4) mg/day (mL)
16	(b) (4)
32	
64	

[1]: [((b) (4) mcg/day)/(Concentration of NVK004, mcg/mL)] = Dose Volume to achieve (b) (4) mg/day

Nevakar submitted NDA 214628 on August 15, 2020 (received August 17, 2020). However, the toxicology study conducted by the Sponsor was not adequate, since the cumulative impurity dose over a period of 28 days in rats was used to qualify the proposed total daily intake (TDI) in human.

The following Information Request (IR) – Nonclinical, was, therefore, submitted on November 23, 2020, and a response received by November 30, 2020.

FDA QUERY

"Regarding the proposed specifications of the two (b) (4) impurities (NMT (b) (4)% for RRT (b) (4) and NMT (b) (4)% for RRT (b) (4)), it is not acceptable to use the cumulative impurity dose over a period of 28 days in rats to qualify the proposed total daily intake (TDI) in human. A higher percentage of the impurity mix can be spiked into the test article in a short-term toxicity

study to qualify the TDI level, or adequate justification (with published literatures), is needed to demonstrate the safety and tolerability of these two impurities at the proposed thresholds."

Sponsor Response

"The 28-Day Repeat Dose Impurity Toxicity GLP Study in Rats ([redacted] Study No. 1602-19287) was designed to emulate the highest level of the two impurities expected in the stability studies based on the maximum tolerated dose (MTD). Since aged material was not available due to the ongoing stability studies, it was decided to spike NVK004 at a specification level equivalent to the TDI and a total exposure to the rats over 28 days (approximately [redacted]-fold longer than a duration of [redacted] days intended [redacted]). In a brief teleconference between the Sponsor and the FDA on November 24, 2020, it was noted that the 28-Day GLP study was a well conducted study and generally acceptable as an evaluation of the proposed formulation, however it does not cover the expected TDI and hence does not provide an adequate safety margin for the two impurities.

It was agreed that the Sponsor would conduct a 14-Day GLP continuous infusion study in rats testing the impurities at least [redacted] the MDD for NVK004 ([redacted] mg/day) corrected for the Human Equivalent Dose (HED) as per FDA guidance (FDA 2005). The study will evaluate vehicle control *vs.* NVK004 (un-spiked) *vs.* NVK004 spiked with the impurities at [redacted] mcg/kg/day for RRT [redacted] ([redacted]% of MDD) and [redacted] mcg/kg/day for RRT [redacted] ([redacted]% MDD) *vs.* vehicle. This study will include the same endpoints as the 28-Day GLP Study ([redacted] No.1602-19287): clinical observations, clinical pathology (hematology, coagulation and clinical chemistry parameters), gross necropsy, including organ weights and a complete list of tissues for histopathologic evaluation. The objective of the submitted studies to review is to determine if the level of Norepinephrine Impurity Mixture (RRT [redacted]/RRT [redacted]) in Nevakar's drug product is safe for human use." The audited draft study report for this 14-day toxicity study was submitted on 4/23/2021 and see page 23 Section 7.5 for its review.

[redacted]

A review of the acceptable studies conducted by the Sponsor is presented below to support the safe use of Norepinephrine Impurity Mixture containing the two impurities: NMT [redacted]% (RRT [redacted]) and NMT [redacted]% (RRT [redacted]) in the Nevakar's drug product (NDA 214628).

7.1. Norepinephrine Impurity Mixture: Bacterial Reverse Mutation Assay

Sponsor:	Nevakar, Inc. Bridgewater, NJ 08807
Protocol No:	P60 1.AMES
Conducting laboratory and location:	(b) (4)
(b) (4) Study No.	52578 (200313-7R)
Date of study initiation:	March 31, 2020
Date of Completion:	May 20, 2020
Drug lot/batch number:	242-TLF-157-1
Purity:	100%
GLP compliance:	Yes
QA statement:	Yes

Key Study Findings

Norepinephrine Impurity Mixture (NVK004) was negative in the Bacterial Reverse Mutation Assay when evaluated for its ability to induce reverse mutations at the histidine locus in strains of *Salmonella* (TA98, TA100, TA1535, and TA1537), and at the tryptophan locus of *E. coli* strain WP2*uvr*A, in the presence or absence of rat metabolic activation system (S9).

Objective:

The objective of this study was to evaluate the mutagenic potential of the Norepinephrine Impurity Mixture to induce gene mutations at selected loci of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system (S9).

Methods

The ability of Norepinephrine Impurity Mixture to induce reverse mutations (Ames et al, 1973) was assessed as per Bacterial Reverse Mutation Assay using the *Salmonella typhimurium strains* (TA98, TA100, TA1535, and TA1537), and *E. coli* (WP2*uvr*A) strain (~ 1 x 10^9 bacteria/mL) at dose level 5000 µg/plate, in the presence or absence of a rat metabolic activation system (S9) and positive control as listed below. The test article was found to be soluble in sterile water, which was used as the vehicle control.

Table 3 1: Bacterial Strains for Mutation Assay

Strain	Characteristics	Mutations Detected	Lot Number	Expiration Date
ST TA98	his; rfa; uvrB; R-factor	Frameshift	5411D	Oct 16, 2021
ST TA100	his; rfa; uvrB; R-factor	Base-pair substitution	5371D	May 30, 2021
ST TA1535	his; rfa; uvrB	Base-pair substitution	5360D	Apr 03, 2021
ST TA1537	his; rfa; uvrB	Frameshift	5350D	Mar 20, 2021
EC WP2 uvrA	trp; uvrA	Base-pair substitution	5351D	Mar 20, 2021

Legend:

his	histidine required as a growth factor
rfa	deep rough mutation involves loss of a major component of the cell coat increasing permeability to larger molecules; this deletion also involves the gene coding for biotin synthesis
uvrA/B	deletion of DNA nucleotide excision repair system
R-factor	contains the pKM101 plasmid which increases sensitivity by enhancing error-prone DNA repair systems
trp	tryptophan required as a growth factor

Table 42: Positive Controls used for Mutation Assay

Positive Control Article Identification	Lot No.	CAS No.	Date of Receipt	(b) (4) ID No.	Expiration Date
Sodium Azide (NaN_3)	6391SA	26628-22-8	Feb 25, 2020	200225-15H	Jan 17, 2022
ICR 191 Acridine	6274ICR	17070-45-0	Feb 25, 2020	200225-17H	Nov 13, 2021
Daunomycin	2181DU	23541-50-6	Feb 25, 2020	200225-18H	Nov 5, 2021
Methyl methanesulfonate (MMS)	8230MS	66-27-3	Feb 25, 2020	200225-16H	Feb 11, 2022
2-Aminoanthracene (2-AA)	6495AA	613-13-8	Feb 25, 2020	200225-14H	Feb 7, 2022

The S9 mix, freshly prepared on the day of use, was maintained on ice prior to and during use and contained 5% v/v S9 fraction.

Table 5 3: Metabolic Activation System

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Criteria for Determination of a Valid Test

The mean revertant colony counts for each strain treated with the vehicle should lie close to or within the expected range considering the laboratory historical control range and/or published values (Mortelmans & Zeiger, 2000; Gatehouse, 2012).

The positive controls (with S9) should produce substantial increases in revertant colony numbers with the appropriate bacterial strain. For each experimental point, the Mutation Factor (MF) was calculated by dividing the mean revertant colony count by the mean revertant colony count for the corresponding concurrent vehicle control group.

The results were considered positive (i.e., indicative of mutagenic potential) if:

- The results for the test item showed a substantial increase in revertant colony counts, i.e., response MF≥ (b) (4) for strains TA98, TAl00, and WP2 uvrA or MF≥ (b) (4) for strains TA1535 and TA 1537, with mean value(s) outside the laboratory historical control range.

The results were considered negative if:

- The increase in revertant colony count is not dose related and/or reproducible, i.e., increases must be obtained at more than one experimental point (at least one strain, more than one dose level, more than one occasion or with different methodologies).

If the second criterion is not met, the results may be classified as equivocal, and further testing

may be appropriate.

For the bacterial strains, plates were prepared for each treatment as follows:

Treatment	Dose No.	Final Dose (µg/plate)	Number of Replicates		Number of Strains
			-S9	+S9	
Vehicle control	0	0	3	3	5
Test article	1	1.58	3	3	5
	2	5.0	3	3	5
	3	15.8	3	3	5
	4	50	3	3	5
	5	158	3	3	5
	6	500	3	3	5
	7	1580	3	3	5
	8	5000[A]	3	3	5
Positive control	*[B]	*[B]	3	3	5

[A] The OECD standard limit dose
[B] Dose depends on the test organism and the positive control

Results
The number of colonies per plate was counted manually and/or with the aid of a plate counter (Colony Plate Reader: Model Colony-Doc-It™). The mean and standard deviation were calculated for each set of triplicate plates.

Toxicity Assay
The results of the toxicity assay conducted at a maximum dose of 5000 µg per plate was achieved using a concentration of 100 mg/mL and a 50.0 µL plating aliquot.

Toxic effects of the impurity mixture are indicated by the partial or complete absence of a background lawn of non-revertant bacteria (colony counts, if any, should not be reported) or a substantial dose-related reduction in revertant colony counts compared with lower dose levels and concurrent vehicle control considering the laboratory historical control range.

There was no cytotoxicity observed for strains T A98, TA 100, TA 1535 and TA 1537 at dose level 5000 µg/plate without S9 in the pre-incubation method.

Mutagenicity Assay
Based upon the results of the preliminary toxicity assay, the dose levels selected for the mutagenicity assay were 50.0, 150, 500, 1500 and 5000 µg per plate. Precipitate was observed at 5000 µg per plate with all conditions.

A moderate toxicity (as a reduction in revertant counts) was observed at 5000 µg per plate with tester strains TA1535 in the absence of S9 activation and TA1537 in the presence of S9 activation. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Table 6: Revertant Colony Counts-TA 98

Pre-Incubation Method - Confirmatory Test									
TA98		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	21 23 21	22	1.2	21 23 22	22	1.0	1.00	1.00
Test Article	1.58	27 20 19	22	4.4	29 26 19	25	5.1	1.00	1.14
Test Article	5	24 25 20	23	2.6	26 25 30	27	2.6	1.05	1.23
Test Article	15.8	18 23 22	21	2.6	30 19 22	24	5.7	0.95	1.09
Test Article	50	22 19 24	22	2.5	19 18 23	20	2.6	1.00	0.91
Test Article	158	26 17 15	19	5.9	25 25 21	24	2.3	0.86	1.09
Test Article	500	22 24 20	22	2.0	29 33 23	28	5.0	1.00	1.27
Test Article	1580	24 24 21	23	1.7	28 31 29	29	1.5	1.05	1.32
Test Article	5000	21/IL 18/IL 22/IL	20	2.1	14 25 28	22	7.4	0.91	1.00
Daunomycin	6	724 848 763	778	63.4	-			35.36	-
2-AA	10	-			3204 2756 3051	3004	227.7	-	136.55

N/A = Not applicable; IL = Incomplete lawn

Table 7: Revertant Colony Counts-TA 100

Pre-Incubation Method - Confirmatory Test									
TA100		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	110 109 111	110	1.0	113 110 111	111	1.5	1.00	1.00
Test Article	1.58	112 99 92	101	10.1	107 106 112	108	3.2	0.92	0.97
Test Article	5	109 112 113	111	2.1	110 99 102	104	5.7	1.01	0.94
Test Article	15.8	99 102 106	102	3.5	112 110 106	109	3.1	0.93	0.98
Test Article	50	91 103 104	99	7.2	105 111 107	108	3.1	0.90	0.97
Test Article	158	100 99 104	101	2.6	108 110 115	111	3.6	0.92	1.00
Test Article	500	118 109 102	110	8.0	110 106 94	103	8.3	1.00	0.93
Test Article	1580	111 96 92	100	10.0	93 112 109	105	10.2	0.91	0.95
Test Article	5000	102/IL 107/IL 110/IL	106	4.0	104 106 111	107	3.6	0.96	0.96
Sodium Azide	1.5	888 902 861	884	20.8	-			8.04	-
2-AA	10	-			3799 2914 3124	3279	462.4	-	29.54

N/A = Not applicable; IL = Incomplete lawn

Table 8: Revertant Colony Counts-TA 1535

Pre-Incubation Method - Confirmatory Test									
TA1535		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	11 13 11	12	1.2	10 12 11	11	1.0	1.00	1.00
Test Article	1.58	13 12 9	11	2.1	11 10 12	11	1.0	0.92	1.00
Test Article	5	8 12 10	10	2.0	12 9 9	10	1.7	0.83	0.91
Test Article	15.8	12 11 11	11	0.6	13 11 10	11	1.5	0.92	1.00
Test Article	50	12 9 8	10	2.1	14 11 9	11	2.5	0.83	1.00
Test Article	158	8 10 10	9	1.2	12 12 12	12	0.0	0.75	1.09
Test Article	500	12 10 9	10	1.5	12 10 10	11	1.2	0.83	1.00
Test Article	1580	10 9 11	10	1.0	12 12 12	12	0.0	0.83	1.09
Test Article	5000	11/IL 12/IL 9/IL	11	1.5	10 11 12	11	1.0	0.92	1.00
Sodium Azide	1.5	800 694 612	702	94.3	-			58.50	-
2-AA	10	-			402 364 421	396	29.0	-	36.00

N/A = Not applicable; IL = Incomplete lawn

Table 9: Revertant Colony Counts-TA 1537

Pre-Incubation Method - Confirmatory Test									
TA1537		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	9 12 12	11	1.7	12 13 10	12	1.5	1.00	1.00
Test Article	1.58	10 9 7	9	1.5	11 10 9	10	1.0	0.82	0.83
Test Article	5	10 8 9	9	1.0	10 7 9	9	1.5	0.82	0.75
Test Article	15.8	10 11 6	9	2.6	10 6 7	8	2.1	0.82	0.67
Test Article	50	11 10 9	10	1.0	10 9 8	9	1.0	0.91	0.75
Test Article	158	11 12 7	10	2.6	10 11 6	9	2.6	0.91	0.75
Test Article	500	10 9 10	10	0.6	10 11 7	9	2.1	0.91	0.75
Test Article	1580	10 11 10	10	0.6	11 10 6	9	2.6	0.91	0.75
Test Article	5000	11/IL 12/IL 9/IL	11	1.5	9 10 11	10	1.0	1.00	0.83
ICR 191 Acridine	1	7460 7449 7348	7419	61.7	-			674.45	-
2-AA	10	-			425 409 363	399	32.2	-	33.25

N/A = Not applicable; IL = Incomplete lawn

Table 10: Revertant Colony Counts- EC WP2 uvrA

Pre-Incubation Method - Confirmatory Test									
E. coli WP2 uvrA		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	39 30 31	33	4.9	32 38 33	34	3.2	1.00	1.00
Test Article	1.58	27 31 25	28	3.1	36 32 38	35	3.1	0.85	1.03
Test Article	5	32 27 29	29	2.5	45 42 34	40	5.7	0.88	1.18
Test Article	15.8	36 28 24	29	6.1	31 36 27	31	4.5	0.88	0.91
Test Article	50	37 24 29	30	6.6	33 40 31	35	4.7	0.91	1.03
Test Article	158	36 32 40	36	4.0	42 29 42	38	7.5	1.09	1.12
Test Article	500	30 29 34	31	2.6	37 34 39	37	2.5	0.94	1.09
Test Article	1580	31 45 39	38	7.0	40 41 38	40	1.5	1.15	1.18
Test Article	5000	42 36 42	40	3.5	39 42 34	38	4.0	1.21	1.12
MMS	2.5[1]	541 610 592	581	35.8	-			17.61	-
2-AA	10	-			88 96 108	97	10.1	-	2.85

N/A = Not applicable

APPENDIX A: PROTOCOL

APPENDIX A: PROTOCOL

The mean revertant colony counts for each strain treated with the vehicle were close to or within the laboratory historical control range. (Mortelmans & Zeiger, 2000; Gatehouse, 2012). The positive control articles caused the expected substantial increases in revertant colony counts in both the absence and presence of S9 in each phase of the test confirming the sensitivity of the test and the activity of the S9 mix.

There was no test article related increases in the number of revertant colonies observed with strains TA98, TA I 00, TA 1535, TA 1537 or *E. coli* WP2 uvrA in both the absence and presence of S9 using either the plate incorporation or the pre-incubation method (data shown) when compared with historical control values. The study seems valid and in compliance with ICH-S2(R1) guidelines.

Conclusion

The results of the Bacterial Reverse Mutation Assay suggest that the Norepinephrine Impurity Mixture (NVK004) did not cause a positive mutagenic response either in the presence or absence of S9 activation system and considered not mutagenic.

7.2. Norepinephrine Impurity Mixture: Mammalian Erythrocyte Micronucleus Test (Peripheral Blood, Flow Cytometry - Mouse)

Sponsor:	Nevakar, Inc. Bridgewater, NJ 08807
Protocol No:	P604.PBF-ME NEV
Conducting laboratory and location:	(b) (4)
(b) (4) Study No.	53153 (200313-7R)
Date of study initiation:	July 2, 2020
Date of Completion:	August 14, 2020
Drug lot/batch number:	242-TLF-157-1
Purity:	100%
GLP compliance:	Yes
QA statement:	Yes

Key Study Findings

In Vivo Mouse Erythrocyte Micronucleus Test (Flow Cytometry) was performed to evaluate the mutagenic potential of Norepinephrine Impurity Mixture to cause damage to the chromosomes or mitotic spindle apparatus of erythroblasts, as an increased micro-nucleated immature erythrocytes (MIE) in mouse peripheral blood.

The results of the *in vivo* Mouse Erythrocyte Micronucleus Test (Flow Cytometry) have shown that there were no significant increase in the frequency of CD71+ reticulocytes (%RET) or micro nucleated immature erythrocytes (%MN-RET) when Norepinephrine Impurity Mixture was administered (IV) at 75 mg/kg/day to male and female mice for two days. The Norepinephrine Impurity Mixture was, therefore not considered to be genotoxic at up to 75 mg/kg/day/IV (a maximum tolerated dose) when compared with positive control.

Objective

The objective of this study was to evaluate the mutagenic potential of the Norepinephrine Impurity Mixture using the *Mammalian Erythrocyte Micronucleus Test* as an increased micro-nucleated immature erythrocyte ((%MN-RET) in mouse peripheral blood.

Methods

The Norepinephrine Impurity Mixture was prepared in 0.9% sterile saline and sterile filtered through a 0.22 µm syringe filter PVDF membrane. The positive control (cyclophosphamide monohydrate prepared in distilled water), the negative control (Sterile saline, 0.9% NaCl) and test article were administered on Days 1 and 2 while the positive control was administered on Day 2 only as indicated below:

Five male and five female test animals were assigned to each of the following test groups.

Group	No. Animals/ Group M/F	Dose Level *(mg/kg body weight)*	Dose Volume (mL/kg body weight/day)	Sampling time (hrs) after final treatment
1	5/5	Negative (Vehicle) Control 0	5	44-48
2	5/5	Test substance (low dose) 18.75		
3	5/5	Test substance (mid dose) 37.5		
4	5/5	Test substance (high dose) 75		
5	5/5	Positive Control (Cyclophosphamide monohydrate) 40		

Five male and five female mice in each group received doses of 0 (vehicle, distilled water) or 18.75, 37.5 and 75 mg/kg/day of Norepinephrine Impurity Mixture (Test Article) for two days, or 40 mg/kg/day of cyclophosphamide monohydrate (positive control) for a single dose.

All animals were observed at least twice daily for viability, and findings were recorded compared to vehicle control (Sterile saline, 0.9% NaCl) as below.

A. Test article

Name (Amendment 5)	Norepinephrine Impurity Mixture
Notebook Reference #	044-KG-186
Composition	(b) (4)
Physical Description	
pH	Not available
Stability	Test article was expected to be stable for the duration of testing. Data provided by the Sponsor showed that the test substance is stable in saline for at least 1 week.
Sterilization	None
Storage	(b) (4)
Retest Date	
CoA	Provided
(b) (4) numbers (Amendment 2)	200625-2R and 200721-1R
Dates Received	June 25, 2020 and July 21, 2020

Appropriate routine safety precautions were exercised in the handling of the test article unless otherwise indicated by the Sponsor. Documentation of the methods of synthesis, fabrication, or derivation of the test article is retained by (b) (4) (b) (4)

B. Positive Control

Name: Cyclophosphamide monohydrate
CAS #6055-19-2
Batch No.: MKCG5464
(b) (4) Reference 181119-2H
Expiration Date: May 2021
Supplier: (b) (4)

The positive control article was stored refrigerated. Documentation of the methods of synthesis, fabrication, or derivation of the test article is retained by (b) (4) (b) (4)

C. Vehicle Control

Name: Sterile saline, 0.9% NaCl
Lot No.: 19D011
Expiration Date: April 2021
Supplier: (b) (4)

At terminal sacrifice, whole blood was collected by cardiac puncture and processed. Cells were stained with dyes to identify immature vs. mature erythrocytes (fluorescent labeled anti-CD71 antibody), platelets (fluorescent labeled anti-CD61 antibody) and DNA (propidium iodide, following RNAse treatment). Stained cells (a minimum of 4000 immature erythrocytes per animal) were analyzed by flow cytometry.

The DNA content (propidium iodide staining) was measured in both CD71+/CD61- (immature erythrocytes) and CD71-/CD61- (mature erythrocytes). [redacted] (b) (4)). The sampling target was 60-120 µL per sample. Duplicate samples were prepared from each mouse. A p-value <0.05 indicated a significant effect.

Criteria for Positive Outcome

The test would be considered positive if at least one of the treatment groups exhibited a statistically significant increase in the frequency of MIEs compared with the concurrent negative control, if the increase(s) were dose-dependent using an appropriate trend test at the time selected for assessment, and if any of the treatment groups had a mean outside of the historical negative control data 95% control limits.

Positive results in the micronucleus test would indicate that the test material induces micronuclei which results from either direct chromosomal damage or damage to the mitotic apparatus in the erythroblasts.

Results

There were no unscheduled mortalities and statistically significant changes in the frequencies of reticulocytes (%RET), micro nucleated normochromatic erythrocytes (%MN-NCE), or micro nucleated immature erythrocytes (%MN-RET/MIEs) compared to vehicle control for either sex when Norepinephrine impurity mixture was administered at doses up to 75 mg/kg/day.

Dose (mg/kg/day)	Number Dead/Number Tested		Mean Peripheral Reticulocyte count ($x10^9$ cells/L)		Mean %Change from Control[1]	
	Males	Females	Males	Females	Males	Females
0 (Vehicle Control)	0/3	0/3	339.1	258.7	-15.0	3.4
75 (MTD)	0/3	0/3	288.1	267.6		

Intravenous treatment of the mice did not result in statistically significant changes to the number of peripheral reticulocytes by paired t-tests.

The results of *Mammalian Erythrocyte Micronucleus Test* using the Norepinephrine impurity mixture, administered at doses up to 75 mg/kg/day to male and female mice and findings are given below.

% RET

Group	Dose (mg/kg/day)	Mean ± SEM		*p*-value	
		Male	Female	Male	Female
Negative (Vehicle) Control	0	1.95 ± 0.31	1.96 ± 0.16	-	-
Test Substance	18.75	1.44 ± 0.10	1.86 ± 0.10	-	-
	37.5	1.99 ± 0.15	2.27 ± 0.23	-	-
	75	1.77 ± 0.08	1.55 ± 0.24	p=0.6282	p=0.3142
Positive Control	40	0.46 ± 0.04	0.51 ± 0.08	p=0.0084*	p=0.0001*

% MN-NCE

Group	Dose (mg/kg/day)	Mean ± SEM		*p*-value	
		Male	Female	Male	Female
Negative (Vehicle) Control	0	0.11 ± 0.01	0.14 ± 0.03	-	-
Test Substance	18.75	0.10 ± 0.00	0.11 ± 0.01	-	-
	37.5	0.12 ± 0.01	0.11 ± 0.01	-	-
	75	0.11 ± 0.01	0.12 ± 0.01	p=0.8928	p=0.5330
Positive Control	40	0.13 ± 0.02	0.14 ± 0.01	p=0.1443	p=1.0000

% MN-RET

Group	Dose (mg/kg/day)	Mean ± SEM		*p*-value	
		Male	Female	Male	Female
Negative (Vehicle) Control	0	0.16 ± 0.01	0.18 ± 0.02	-	-
Test Substance	18.75	0.13 ± 0.01	0.13 ± 0.01	-	-
	37.5	0.15 ± 0.02	0.15 ± 0.02	-	-
	75	0.14 ± 0.02	0.18 ± 0.02	p=0.5621	p=0.8310
Positive Control	40	1.60 ± 0.30	1.39 ± 0.23	p=0.0074*	p=0.0009*

- = not tested
* = $p < 0.05$ indicates the comparison is statistically significant compared to the negative control using paired t-tests.

% RET = frequency (%) of CD71 positive reticulocytes
% MN-NCE = frequency (%) of micronucleated normochromatic erythrocytes
% MN-RET = frequency (%) of positive CD71 micronucleated reticulocytes (i.e., micronucleated immature erythrocytes [MIEs])

Conclusion

The results of *Mammalian Erythrocyte Micronucleus Test* demonstrate that Norepinephrine impurity mixture, administered at doses up to 75 mg/kg/day to male and female mice did not induce a statistically significant changes in the frequencies of reticulocytes (%RET), micro nucleated normochromatic erythrocytes (%MN-NCE), or micro nucleated immature erythrocytes (%MN-RET/MIEs).

Based on the results of *Mammalian Erythrocyte Micronucleus Test*, Norepinephrine Impurity Mixture (NVK004) is not considered to be genotoxic.

7.3. *In vitro* Mammalian Chromosome Aberration Test in Chinese Hamster V79 Cells with NVK004 Impurity Mixture

Sponsor:	Nevakar, Inc. Bridgewater, NJ 08807
Protocol No:	NVK004TXXN0057
Conducting laboratory and location:	(b) (4)
(b) (4) Study No:	(b) (4) 20AA1089-2
Date of study initiation:	April 8, 2020
Date of Completion:	May 12, 2020
Purity:	98%
GLP compliance:	Yes
QA statement:	Yes

Key Study Findings

Results of an *in vitro* chromosomal aberration test performed in Chinese Hamster V79 cells to induce the structural chromosomal aberrations by Norepinephrine Impurity Mixture (NVK004), have shown a statistically significant increase ($p < 0.05$) in aberrant cells when tested at a concentration of 300 µg/mL and higher in without metabolic activation system and at concentration 400 µg/mL and higher with metabolic activation system. The data, therefore, suggest that NVK004 Impurity Mixture is clastogenic at a concentration of 300 µg/mL and higher in inducing structural chromosomal aberrations in the V79 Chinese Hamster cell line.

However, considering the maximum daily dose of (b) (4) mg norepinephrine, the maximum conceivable blood concentration of impurities, would be (b) (4) mcg/mL which is between (b) (4) and (b) (4)-fold less than the concentration where clastogenicity was observed ((b) (4) and (b) (4) mcg/mL without and with activation, respectively.

Objective

The objective of this study was to evaluate the clastogenic potential of NVK004 Impurity Mixture to induce structural chromosome aberrations in Chinese Hamster V79 cells, an *in vitro* chromosome aberration assay.

Methods

The Norepinephrine Impurity Mixture and the positive control (EMS; ethylmethanesulfonate) were prepared in dissolving and diluting the test substance in cell culture medium within 1 hour prior to treatment. while the treatment medium was considered as negative control in this experiment.

Complete Culture Medium

MEM medium supplemented with:

10	% (v/v)	fetal bovine serum (FBS)
100 U/100	µg/mL	penicillin/streptomycin solution
2	mM	L-glutamine
2.5	µg/mL	amphotericin
25	mM	HEPES

Also used for the long-term treatment and the post incubation.

Treatment Medium (short-term exposure)

Complete culture medium without FBS.

The cofactors in the S9 mix (5% v/v) were prepared as below:

8	mM	$MgCl_2$
33	mM	KCl
5	mM	Glucose-6-phosphate
5	mM	NADP

in 100 mM sodium-phosphate-buffer pH 7.4. During the experiment the S9 mix was stored on ice.

Table. 11: The experimental design

Preparation day 1	Seeding of the cells in 25 cm² culture flasks
1. Day of the Test: Incubation (approx. 48 h after seeding of the cells)	The culture medium was replaced by serum-free medium (short-term incubation) or by 10% serum-containing medium (long-term treatment) containing different concentrations of the test item and S9 mix (only with metabolic activation). Beginning of the treatment Incubation for 4 h for short-term treatment and 21 h for long-term treatment. Additional negative and/or solvent and positive controls were treated in the same way. Cytotoxicity and precipitation was determined after the treatment period of the cultures.
2. Day of the Test: Preparation of the Cultures	Colcemid was added to the cultures of approximately 2 h before the preparation. The cultures were harvested 21 h after beginning of treatment. After centrifugation, the supernatant was discarded and cells were resuspended in approximately 7 mL hypotonic solution (0.4 % KCl). After removal of the hypotonic solution by centrifugation cells were fixed with methanol/glacial acetic acid (3:1, v/v). The fixation procedure was done at least two times and afterwards cells were spread onto slides.
3. Day of the Test: Staining of the Cells with Giemsa	The air dried slides were stained with Giemsa solution.
Analysis of Metaphase Cells	All structural chromosome aberrations such as breaks, fragments, deletions, exchanges and chromosomal disintegration were recorded. Gaps were recorded as well but are not included in the calculation of the aberration rates.
Relative Increase in Cell Count	Cytotoxicity was assessed by the relative increase in cell count. Values were compared with negative/solvent controls.

Acceptability of the Assay

The chromosomal aberration assay is considered acceptable if it meets the following criteria:

a. the number of aberration found in the negative and/or solvent controls falls within the range of historical laboratory control data / is considered acceptable for addition to the laboratory historical negative control database.
b. concurrent positive controls should induce responses that are compatible with those generated in the historical positive control data base and produce a statistically significant increase compared with the concurrent negative control
c. the proliferation criteria in the solvent control should be like the corresponding negative control (where applicable)
d. all three experimental conditions were tested unless one resulted in positive results
e. adequate number of cells and concentrations are analyzable
f. the criteria for the selection of top concentration are consistent with those described earlier

Evaluation of Results

Providing that all acceptability criteria are fulfilled, a test chemical is clearly positive if, in any of the experimental conditions examined:

a) at least one of the test concentrations exhibits a statistically significant increase compared withthe concurrent negative control

b) the increase is dose-related when evaluated with an appropriate trend test
c) any of the results are outside the 95% control limits of the historical negative control data.

When all these criteria are met, the test chemical is then considered to be able to induce chromosomal aberrations in cultured mammalian cells in this test system.

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control
b) there is no concentration-related increase when evaluated with an appropriate trend test
c) all results are inside the 95% control limits of the historical negative control data.

The test chemical is then considered unable to induce chromosomal aberrations in cultured mammalian cells in this test system.

Results
Based on the toxicity data following concentrations were evaluated for microscopic analysis:

without metabolic activation: 100, 300 and 500 µg/mL
with metabolic activation: 300, 400 and 500 µg/mL

Data from the cytotoxicity and aberration experiments are shown below:

Table 12: Summary of Cytotoxicity Experiment

Dose Group	Concentration [µg/mL]	Cell Count Culture 1	Culture 2	Mean	RICC [%]	Precipitate (+/-)
without metabolic activation						
C	0	154.45	181.91	168.18	100	-
2	100	211.26	185.69	198.48	119	-
4	300	118.46	98.49	108.48	62	-
6	500	113.73	104.26	108.99	63	-
EMS	900	143.08	148.76	145.92	86	-
with metabolic activation						
C	0	178.12	148.76	163.44	100	-
4	300	154.45	131.72	143.08	87	-
5	400	93.75	108.99	101.37	60	-
6	500	120.36	99.44	109.90	65	-
CPA	1.11	156.34	142.14	149.24	91	-

RICC: Relative Increase in Cell Count, calculated by the increase in cell number of the test groups compared to the negative control. The cell count was determined by a cell counter per culture for each test group.

C: Negative Control (Culture Medium)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)
CPA: Positive Control (with metabolic activation: Cyclophosphamide)

The cytotoxic effect determined as the relative increase in cell count (RICC) was determined.

The RICC was calculated as follows:

$$RICC\ (\%) = \frac{N\text{-}N_0\ (treated)}{N\text{-}N_0\ (untreated)} \times 100$$

N_0: initial cell number; N: cell number at end of treatment

Additionally the number of polyploid cells is scored. Polyploid means a near tetraploid karyotype in the case of this aneuploid cell line.

Table 13: Summary of Aberration Experiment

Dose Group	Concentration [µg/mL]	Treatment Time	Fixation Interval	mean % aberrant cells		Precipitation
				incl. Gaps	excl. Gaps	
without metabolic activation						
C	0	4	21	4.7	2.0	-
2	100	4	21	4.7	2.0	-
4	300	4	21	7.7	6.0	-
6	500	4	21	9.1	5.4	-
EMS	900	4	21	11.6	11.1	-
with metabolic activation						
C	0	4	21	4.3	3.3	-
4	300	4	21	5.3	4.3	-
5	400	4	21	8.0	7.3	-
6	500	4	21	9.2	7.7	-
CPA	1.11	4	21	12.8	11.2	-

300 cells evaluated for each concentration, except for the positive controls EMS (225 cells) and CPA (250 cells) due to a clearly positive increase in chromosomal aberrations. In the experiment without metabolic activation, more than 300 cells were evaluated for chromosome aberrations for dose group 6 (573 cells). In the experiment with metabolic activation only 260 metaphases were evaluated for the concentration of 500 µg/mL.

C: Negative Control (Culture Medium)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)
CPA: Positive Control (with metabolic activation: Cyclophosphamide)

The aberration rate of the negative control (2.0%) in **without** metabolic activation experiment was within the historical control data (0.02 to 3.76% aberrant cells).

No increase of aberrant cells was observed at 100 µg/mL (2.0%). An increase of aberrant cells was observed in the treated dose groups at concentrations of 300 µg/mL(6.0%) and 500 µg/mL (5.4%). These increases were statistically significant compared to the concurrent negative control.

The aberration rate of the negative control (3.3%) in **with** metabolic activation was within the historical control data of the testing facility (-0.32 to 3.54% aberrant cells exclusive gaps). An increase of aberrant cells above the upper historical control limit was observed in the treated dose groups at all concentrations.

The aberration rates were 4.3% (300 µg/mL), 7.3% (400 µg/mL) and 7.7% (500 µg/mL). At 400 and 500 µg/mL these increases were statistically significant compared to the concurrent negative control.

Conclusion
The data from chromosomal aberration test in the V79 Chinese Hamster cell line suggest that NVK004 Impurity Mixture induced structural chromosomal aberrations and is clastogenic at a concentration of 300 µg/mL and higher (at a concentrations ≥300 µg/mL impurity mixture without S9 activation and ≥400 µg/mL impurity mixture with S9 activation).

Considering the maximum daily dose of norepinephrine at (b) (4) mg the maximum blood concentration (Cmax) of NVK004 Impurity Mixture would be ~ (b) (4) mcg/mL, which is at least (b) (4)-fold less than the concentration that showed clastogenic activity.

Since clastogenicity can be considered a threshold event, this finding is not considered clinically relevant to the drug product (Norepinephrine in Sodium Chloride Injection), as the clastogenic effect occurred at extremely high concentrations compared to potential human exposure ((b) (4)-fold or higher) and was negative at lower concentrations.

The relative risk is even lower for this drug product, indicated for short duration of use. In addition, the in vivo micronucleus assay was negative and therefore it was concluded that the two impurities are not considered genotoxic at clinically relevant dose range.

7.4. Determining the Hemolytic Potential of the Sponsor's test Article at Four Concentrations in Fresh Un-clotted Blood (Rat/Dog/Monkey/Human)

Sponsor:	Nevakar, Inc. Bridgewater, NJ 08807
Study No.:	NVK004IVXN-0002
Conducting laboratory and location:	(b) (4)
Study No:	16NEVAP1R1
Date of Completion:	December 23, 2016
GLP compliance:	No
QA statement:	No

Key Study Findings
Hemolytic potential of NVK004 impurity mixture was tested in fresh whole un-clotted blood of rat, dog, cynomolgus monkey, and human. The hemolytic criteria set for the hemolysis of excipients (Amin and Dannenfelser, 2006) the hemolysis observed up to 10% is considered non-hemolytic. Based on the results (9% hemolysis) of this study, NVK004 impurity mixture is therefore, considered non-hemolytic to red blood cells in rat, dog, cynomolgus monkey or human blood.

Objective
The objective of this study was to evaluate the hemolytic potential of NVK004 Impurity Mixture in fresh whole un-clotted blood of rat, dog, Cynomolgus monkey, and human.

Methods
Blood samples were received on wet ice with overnight delivery and used the day of delivery within 24 hours of blood collection. Sodium citrate was added to blood samples on the site of collection as per standard protocol (Amin and Dannenfelser, 2006) described below:

Experimental Details

Materials and Equipment	Distributor	Cat #	Lot #
Human whole blood (Na citrate anticoagulant)	(b) (4)	HMWBCIT	BRH1247407
Human whole blood (Na citrate anticoagulant)		HMWBCIT	BRH1249112
Beagle dog whole blood (male) (Na citrate anticoagulant)		BGLWBCIT-M	BGL91217
Beagle dog whole blood (male) (Na citrate anticoagulant)		BGLWBCIT-M	BGL91306
Cynomolgus monkey whole blood (male) (Na citrate anticoagulant)		CYNWBCIT-M	CYN195406-CYN195407
Rat whole blood (male) (Na citrate anticoagulant)		RATWBCIT-M	RAY313141-RAY313142
0.9% NaCl (NS)		510223	NA
10% Na dodecylsulfate (SDS) solution in water (10% SDS solution was diluted to 2% SDS solution with deionized water)		V6553	0000049864
Spectrophotometer			
Reciprocal shaking water bath			
Centrifuge			

Two controlled solutions, Sodium Chloride (0.9%) and SDS (2%) in water were used as 0% and 100% hemolytic solutions, respectively. The 0.9 mL of fresh whole un-clotted blood from rat, dog, Cynomolgus monkey, and human), containing Na citrate as an anticoagulant, was mixed with 0.1 mL of dosing or controlled solution in triplicate, and incubated for 45 minutes.

Hemolytic Procedure

1. Prepare three identical sets of for each sample: 0.9 mL of whole blood (citrated) mixed with 0.1 mL of test solution.
2. In parallel, set 2 controls NS and 2% SDS in water as a 0% hemolysis and 100% hemolysis.
3. Agitate gently for 2-3 seconds.
4. Incubate at 37°C for 45 minutes.
5. Quench the reaction at assigned time by adding 5 mL of NS.
6. Centrifuge for 5 minutes at 3000 rpm.
7. Discard 4.5 mL of the supernatant and replace it with 4.5 mL of NS; vortex.
8. Centrifuge for 5 minutes at 3000 rpm.
9. Repeat procedures in Steps 7 and 8 three times.
10. Discard the supernatant and add an aliquot of 5 mL sterile water to the remaining plug, vortex, and centrifuge for 5 minutes at 3000 rpm to release the hemoglobin from the intact cells.
11. Use supernatant for analysis to read the absorbance at 540 nm.
12. The ABS is proportional to amount of intact RBCs.
13. Calculate the % of hemolysis.

$$\%\text{Hemolysis} = (ABS_{NS} - ABS_{TC})*100/(ABS_{NS} - ABS_{SDS})$$

Where ABS_{NS}: absorbance at 540 nm for NS/blood samples (no lysis, max number); absorbance at 540 nm for TC/blood; absorbance at 540 nm for SDS (total lysis, min number)
*When % hemolysis is a negative number it is reported as 0.

Results

The hemolytic reaction was quenched by adding 5 mL of Sodium Chloride (0.9%). All samples were centrifuged for 5 minutes at 3000 rpm to discard released hemoglobin. The procedure was repeated three times followed by adding 5 mL of de-ionized water, centrifugation and measuring hemoglobin concentration in the final supernatant. Each supernatant was diluted 22-fold prior to measuring absorbance at 540 nm.

Table: Detection of Hemolysis in Whole Blood of Rat (Male), Beagle Dog (Male), Cynomolgus Monkey and Human

Test Formulation 1	% Hemolysis			
Species	RD007-137 A	RD007-137 B	RD007-137 C	RD007-137 D
Rat Blood	0	1	6	0
Dog Blood	0	2	2	0
Monkey Blood	5	2	8	7
Human Blood	4	2	3	0
Test Formulation 2	% Hemolysis			
Species	RD007-139 A	RD007-139 B	RD007-139 C	RD007-139 D
Rat Blood	0	0	0	0
Dog Blood	2	3	2	3
Monkey Blood	0	0	3	0
Human Blood	9	2	5	0
Test Formulation 3	% Hemolysis			
Species	RD007-141 A	RD007-141 B	RD007-141 C	RD007-141 D
Rat Blood	7	0	8	0
Dog Blood	0	0	4	0
Monkey Blood	4	2	9	5
Human Blood	0	1	0	0
Test Formulation 4	% Hemolysis			
Species	RD007-143 A	RD007-143 B	RD007-143 C	RD007-143 D
Rat Blood	7	1	0	0
Dog Blood	0	0	0	0
Monkey Blood	2	0	0	0
Human Blood	7	1	0	3

Test Formulation 4 (pH = 5.0)	**% Hemolysis**			
Species	**RD007-143 A (16 mcg/mL)**	**RD007-143 B (32 mcg/mL)**	**RD007-143 C (64 mcg/mL)**	**RD007-143 D (160 mcg/mL)**
Rat Blood	7	1	0	0
Dog Blood	0	0	0	0
Monkey Blood	2	0	0	0
Human Blood	7	1	0	3

The data from the hemolytic experiments have shown that the maximum hemolysis observed (9%) in the whole blood samples of whole blood of rat beagle dog , cynomolgus monkey and human as a result of the exposure of NVK004 impurity mixture lies within the nonhemolytic criteria of 10% as below:

Table: Criteria for Hemolysis

Percent Hemolysis	**Interpretation**
<10%	Not Hemolytic
10%-25%	Relative Boundary (Possibly Hemolytic)
>25%	Hemolytic

Conclusion

Based on the criteria set for hemolytic potential in whole blood (Amin and Dannenfelser, 2006) the test article NVK004 (Norepinephrine in Sodium Chloride Injection solution) is considered not hemolytic in rat, dog, Cynomolgus monkey or human blood in vitro.

7.5. NVK004 Impurities: A 14-Day Repeat Dose Toxicity Study Following Continuous Intravenous Administration to Sprague Dawley Rats

Sponsor:	Nevakar, Inc. Bridgewater, NJ 08807
Conducting laboratory and location:	(b) (4)
(b) (4) Study No.	1602-20391
Date of study initiation:	January 12, 2021
Date of Draft Report Submission:	April 21, 2021
NVK004 lot/batch number:	1980008
Purity:	99.9%
GLP compliance:	Yes
QA statement:	Yes (audited draft report)

Key Study Findings

NVK004 administration alone or spiked with the impurities at (b) (4) or (b) (4) the maximum daily dose resulted in decreased body weight and increase in BUN in all treated groups. NVK004 also resulted in adverse effects on the heart, arteries and arterioles (multiple organs), lungs, and kidneys in all treated groups that were adverse and were attributed to the exaggerated pharmacologic effects of NVK004 on the cardiovascular system.

Objective

The objective of this study was to determine the potential toxicity of NVK004 impurities (RRT (b) (4) and RRT (b) (4)) when administered for 14 days by continuous intravenous administration to male and female rats.

Methods

The dose formulations for control sample (0.9% NaCl and 0.01% EDTA, pH 4: Group 1)), test article, NVK004 (un-spiked: Group 2), NVK004 spiked at (b) (4) MDD(Group 3) and NVK004 spiked at (b) (4) MDD (Group 4) were prepared and stored as below:

Sample Type	Concentrations Sampled	Stratum	Sample Volume (mL)	Number of Samples per Concentration			Intervals Sampled	Storage Conditions
				Collected	Analyzed	Backup		
Stability	Groups 2-4	Middle	2.0	4	2	2	Mix 1, SD 1	5 ± 3°C
Concentration Verification	All (includes control)	Middle	2.0	4	2	2	Mixes 1-3, at time of formulation	

The dose levels for the impurities (RRT (b) (4) and RRT (b) (4) impurity specifications of (b) (4)% and (b) (4)%, respectively) were selected as equivalent to receive by humans at (b) (4) and (b) (4) the Maximal Daily Dose (MDD, (b) (4) mg) corrected for species allometric scaling ((b) (4)).

Formulations were equilibrated to room temperature for at least 30 minutes prior to infusion start and all animals were dosed by continuous intravenous infusion administration into the vena cava via a catheter surgically implanted into the femoral vein.

The volume of administration was adjusted to provide a similar dose (µg/kg/day) to that of the females and males as per following study design.

Group	Treatment	Nominal Norepinephrine Doses (µg/kg/day)	Nominal Impurity (b) (4) (RRT (b) (4) Dose (µg/kg/day)	Nominal Impurity (b) (4) (RRT (b) (4) Dose (µg/kg/day)	Number of Animals Main Phase	
					Males	Females
1	Control	0	0	0	10	10
2	NVK004 (unspiked)	920	0	0	10	10
3	NVK004 (spiked at (b) (4) MDD)	920	45.7	21.1	10	10
4	NVK004 (spiked at (b) (4) MDD)	920	91.3	42.2	10	10

Control = 0.9% sodium chloride 0.01% EDTA, pH 4

Group	Treatment	Main Phase Animals	
		Males	Females
1	Control	14774–14783	14784–14793
2	NVK004 (unspiked)	14794–14803	14804–14813
3	NVK004 (spiked at (b) (4) MDD)	14814–14823	14824–14833
4	NVK004 (spiked at (b) (4) MDD)	14834–14843	14844–14853

All animals were observed as per following specification:

Procedure	Frequency
Cageside Observations (mortality, moribundity, and general health)	≥ twice daily
Physical Examinations (skin and fur characteristics, eye and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor and behavior patterns, and surgical sites)	Study Day 1 (prior to initiation of dosing) Weekly thereafter Prior to necropsy
Body Weights	SD1 (prior to initiation of dosing) Weekly thereafter Day prior to necropsy (unfasted) Prior to necropsy (fasted)
Food Consumption – Quantitative	Weekly starting on SD 1
Ophthalmologic Examinations	Prior to randomization and on SD 12

Animals were fasted overnight (with water available) and blood samples for Clinical Pathology were collected from all surviving animals as below:

Parameter	Chemistry	Hematology	Coagulation
Collection Day	Prior to necropsy (SD 15)		
Collection Method	Cardiac puncture under 70% CO_2/30% O_2 anesthesia		
Volume Collected	at least 1 mL	at least 0.5 mL	at least 1.8 mL
Tubes Used	serum separator	K_2 EDTA	sodium citrate

Clinical Chemistry analysis was performed as below:

Parameter	Abbreviation	Units	Method
Albumin	ALB	g/dL	Bromcresol green (colorimetric)
Alkaline Phosphatase	ALPi	U/L	p-Nitrophenyl-phosphate (bichromatic rate)
Alanine Aminotransferase	ALTi	U/L	L-Alanine to Alpha Ketoglutarate (bichromatic rate)
Aspartate Aminotransferase	AST	U/L	L-Aspartate to Alpha Ketoglutarate (bichromatic rate)
Blood Urea Nitrogen	BUN	mg/dL	Urease (bichromatic rate)
Calcium	CA	mg/dL	o-cresolphthalein complexone (colorimetric)
Cholesterol	CHOL	mg/dL	Cholesterol Esters (colorimetric)
Creatine Kinase	CK	U/L	Creatine phosphate to Adenosine-Diphosphate (multiple point rate)
Chloride	CL	mmol/L	Ion-selective electrode (potentiometric)
Creatinine	CRE2	mg/dL	Creatinine + Picrate (bichromatic rate)
Gamma Glutamyltransferase	GGT	U/L	gamma-glutamyl-3-carboxy-4-nitroanilide to glycylglycine (bichromatic rate)
Glucose	GLU	mg/dL	Hexokinase-Glucose-6-Phosphate Dehydrogenase (colorimetric)
Potassium	K	mmol/L	Ion-selective electrode (potentiometric)
Sodium	NA	mmol/L	Ion-selective electrode (potentiometric)
Phosphorus	PHOS	mg/dL	Phosphomolybdate (colorimetric)
Total Bilirubin	TBILI	mg/dL	Diazo (colorimetric)
Total Protein	TPROT	g/dL	Biuret (colorimetric)
Triglycerides	TRIG	mg/dL	Glycerol-3-phosphate (colorimetric)

Globulin (GLOB, g/dL) was calculated as the difference between total protein and albumin. The albumin to globulin ratio (A/G) was obtained by dividing the albumin value by the globulin value. Values that were below the detection limit of the analyzer were reported as < the lowest limit of the measured parameter.

The lowest limit for triglyceride concentration determination is 15 mg/dL. A value that was observed to be less than this was reported as <15.

Hematology analysis was performed as below:

Parameter	Abbreviation	Units	Method
Complete Blood Count			
White Blood Cells	WBC	K/μL	Flow cytometry by 2 angles of light scatter Low angle: number of nuclei present High angle: nuclear complexity
Red Blood Cells	RBC	M/μL	Flow cytometry by 2 angles of light scatter Low angle: size High angle: hemoglobin concentration
Hemoglobin	HGB	g/dL	Measured by cyanmethemoglobin
Hematocrit	HCT	%	Calculated: (RBC x MCV) ÷ 10
Mean Corpuscular Volume	MCV	fL	Mean of RBC volume histogram
Mean Corpuscular Hemoglobin	MCH	pg	Calculated: (HGB ÷ RBC) x 10
Mean Corpuscular Hemoglobin Concentration	MCHC	g/dL	Calculated: (HGB ÷ [RBC x MCV]) x 1000
Mean Platelet Volume	MPV	fL	Flow cytometry by low angle light scatter
Platelets	PLT	K/μL	Flow cytometry by 2 angles of light scatter Low angle: size High angle: refractive index
Red Cell Distribution Width	RDW	%	Calculated: 100 x (SD of RBC volume histogram ÷ MCV)

The coagulation test was performed as below:

Parameter	Abbreviation	Units	Method
Prothrombin Time	PT	seconds	Laser-nephelometric centrifugal analyzer
Activated Partial Thromboplastin Time	APTT	seconds	Laser-nephelometric centrifugal analyzer
Fibrinogen	FIB	mg/dL	Laser-nephelometric centrifugal analyzer based on the consumption of fibrinogen in the PT assay

Termination, Necropsy, and Histopathology

All surviving animals were euthanized on the day 15 by carbon dioxide inhalation followed by exsanguination prior to necropsy.

Animals were necropsied after the time of death or discovery. Bone marrow smears were prepared, organs were weighed, and protocol-specified tissues were collected and preserved.

Preserved tissues from all animals in Groups 1, 2, and 4, and from each animal found dead (regardless of group) were embedded in paraffin, sectioned, stained with hematoxylin and eosin, examined as shown below.

Organ Name	Organ Weights	Collected in 10% NBF	Microscopic Examination
Artery, Aorta		X	X
Joint, Femorotibial		X	X
Bone, Sternum		X	X
Bone, Marrow		X	X
Brain	X	X	X[2]
Cervix		X	X
Epididymis	X[3]	X[1,4]	X[1]
Esophagus		X	X
Eye		X[1,4]	X[1]
GALT		X	X
Gland, Adrenal	X[3]	X[1]	X[1]
Gland, Harderian		X[1]	X[1]
Gland, Mammary		X	X[5]
Gland, Pituitary	X	X	X
Gland, Prostate		X	X
Gland, Salivary, Mandibular		X[1]	X[1]
Gland, Seminal Vesicle		X[1]	X[1]
Gland, Coagulating gland		X[1]	X[1]
Gland, Thyroid	X[3] *(with parathyroid)*	X[1]	X[1]
Gland, Parathyroid		X	X[6]
Heart	X	X	X
Identification		X	-
Infusion Site (including catheter tip)		X	X
Kidney	X[3]	X[1]	X[1]
Large Intestine, Cecum		X	X
Large Intestine, Colon		X	X
Large Intestine, Rectum		X	X
Lesion(s)		X	X
Liver	X	X	X
Lung		X[1]	X[1]
Lymph Node, Mandibular		X[1]	X[1]
Lymph Node, Mesenteric		X	X
Muscle, Biceps Femoris		X	X
Nerve, Optic		X[1,4]	X[1]
Nerve, Sciatic		X	X
Ovary	X[3] *(with oviduct)*	X[1]	X[1]
Oviduct		X[1]	X[1]
Pancreas		X	X
Skin		X	X
Small Intestine, Duodenum		X	X
Small Intestine, Ileum		X	X
Small Intestine, Jejunum		X	X
Spinal Cord, Cervical		X	X
Spinal Cord, Thoracic		X	X
Spinal Cord, Lumbar		X	X
Spleen	X	X	X
Stomach		X	X
Testis	X[3]	X[1,4]	X[1]
Thymus	X	X	X
Trachea		X	X
Urinary bladder		X	X
Uterus	X *(with cervix)*	X[1]	X[1]
Vagina		X	X

[1] Paired or bi-lobed organ with both collected and examined microscopically
[2] Six levels evaluated; adapted from Bolon et al., 2013
[3] Paired organs weighed together
[4] Collect in modified Davidson's solution and subsequently transferred to NBF except for found dead animals.
[5] For males, examined if in the plane of section; histologic rework not required if not in the plane of section
[6] One of a pair is sufficient for microscopic examination

Statistical Analysis

Quantitative data (body weights, body weight changes, food consumption, clinical pathology, and organ weight data) from the treated groups were compared statistically to the data of the control group using One Way Analysis of Variance (ANOVA) techniques; sexes were analyzed separately.

Prior to the ANOVA analysis, untransformed data were tested to determine if the data are normally distributed and have homogeneous variances among all groups.

The Dunnett's t-test was used to determine which groups (if any) differed from the control group. Group comparisons were evaluated at the 0.05 (two-tailed) probability level. Statistical significance at $p \leq 0.05$ was used throughout the text of the report to indicate if the values were significant.

Results

NVK004 treatment did not affect physical examinations or cage-side observations. There were no mortalities observed as a result of NVK004 administration in rats. A single male in Group 3 was found dead on SD 15 (day of necropsy).

The cause of death was not attributed to test article-related because there was no additional mortality observed in any group treated with NVK004 and impurities at (b) (4) the maximum daily dose.

Body Weight Changes

The mean absolute body weight changes were significantly lower in NVK004 (NVK004 alone or spiked with the impurities at (b) (4) or (b) (4) the maximum daily dose) treated groups when compared with control for both males and females.

The decrease in relative body weight (as shown below in Figure 1 and 2) was correlated with the significant decrease in total food consumption. The decrease in body weight gain in males was more significant with group 3 (spiked with (b) (4) impurity mix) and group 4 (spiked with (b) (4) impurity mix) than group 2 (drug product alone). In females, group 2-4 all showed a decrease in absolute body weight at the end of the dosing period, which was more significant in group 2.

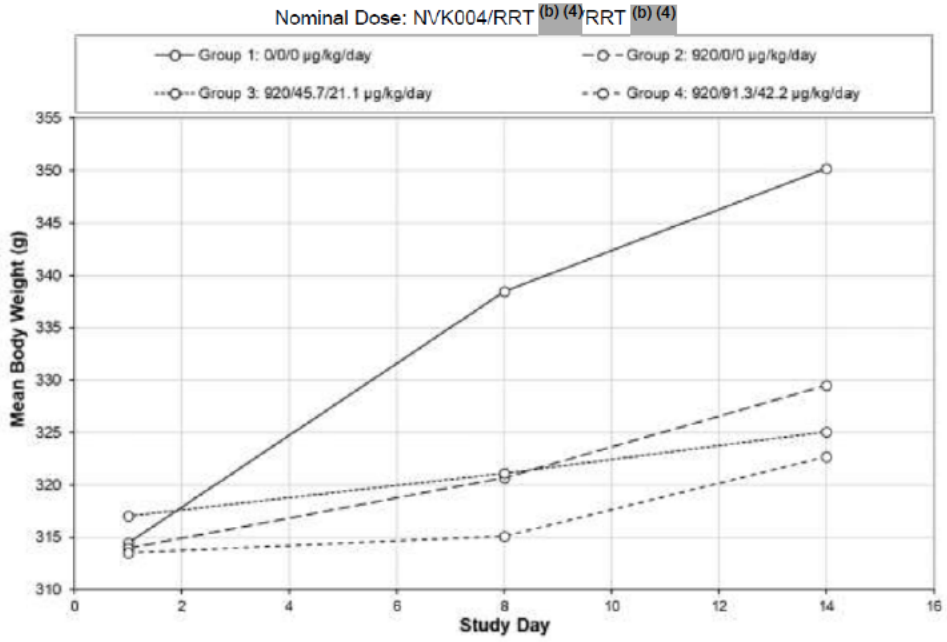


Fig. 1. Changes in Mean Body Weight in male SD rats following NVK004 impurities administration.

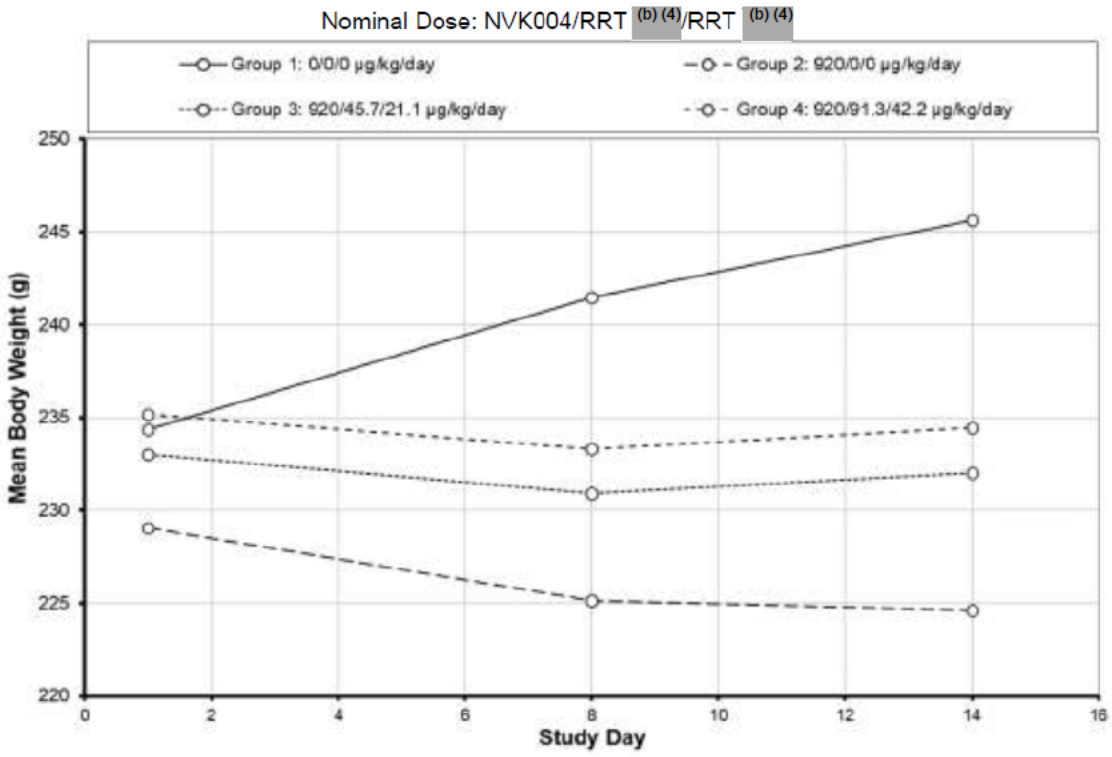


Fig. 2. Changes in Mean Body Weight in female SD rats following NVK004 impurities administration.

There were no ophthalmology related changes or ocular lesions seen in any animals treated with NVK004 alone or spiked with the impurities at (b) (4) or (b) (4) the maximum daily dose.

Clinical Chemistry

There were mild increases in mean BUN (up to 1.57x), relative to controls, on day 15 in Groups 2 (nominal 920/0/0 µg/kg/day), 3 (nominal 920/45.7/21.1 µg/kg/day), and 4 (nominal 920/91.3/42.2 µg/kg/day) in both sexes indicative of decreased glomerular filtration rate (GFR), which seem to be consistent with microscopic findings of glomerulopathy in these groups. The magnitude of changes was similar among group 2, 3 and 4, indicating this is likely due to the administration of NVK004, but not related to the impurities.

Day 15 Relative to Start Date

Group	Sex		GLU mg/dL	BUN mg/dL	CRE2 mg/dL	NA mmol/L	K mmol/L	CL mmol/L	CA mg/dL	PHOS mg/dL	CHOL mg/dL	TRIG mg/dL
1	m	Mean	107.8	20.7	0.49	145.9	5.92	105.3	11.80	10.14	121.5	44.3
		S.D.	12.0	2.1	0.07	1.3	0.42	0.7	0.44	1.05	18.8	11.6
		N	10	10	10	10	10	10	10	10	10	10
2	m	Mean	108.8	27.6*	0.50	145.7	6.14	104.4	12.10	11.05	146.4*	40.9
		S.D.	23.8	5.0	0.07	0.9	0.63	1.0	0.55	1.00	21.9	12.5
		N	10	10	10	10	10	10	10	10	10	10
3	m	Mean	117.7	27.8*	0.51	145.6	5.83	104.8	12.51*	11.39*	139.8	38.7
		S.D.	25.0	3.2	0.06	0.7	0.68	0.8	0.28	0.72	10.7	14.2
		N	9	9	9	9	9	9	9	9	9	9
4	m	Mean	115.6	28.1*	0.53	146.7	6.08	104.6	11.99	10.70	141.3*	42.8
		S.D.	28.0	4.6	0.05	1.4	0.41	1.0	0.24	1.01	17.0	17.0
		N	10	10	10	10	10	10	10	10	10	10

* - Significantly different from the control value, p ≤ 0.05

Nominal Dose (NVK004/RRT (b) (4)/RRT (b) (4): Group 1- 0/0/0 µg/kg/day Group 2- 920/0/0 µg/kg/day
Group 3- 920/45.7/21.1 µg/kg/day Group 4- 920/91.3/42.2 µg/kg/day

Day 15 Relative to Start Date

Group	Sex		TPROT g/dL	ALB g/dL	GLOB g/dL	A/G ratio	AST U/L	ALTi U/L	ALPi U/L	TBILI mg/dL	GGT U/L	CK U/L
1	m	Mean	6.87	3.10	3.77	0.86	103.4	74.2	137.9	0.16	4.3	229.8
		S.D.	0.16	0.71	0.76	0.24	15.1	4.7	16.5	0.05	0.5	83.5
		N	10	10	10	10	10	10	10	10	10	10
2	m	Mean	6.94	3.24	3.70	0.88	123.9	91.2*	163.4*	0.13	4.7	301.4
		S.D.	0.37	0.18	0.22	0.04	32.5	9.2	28.6	0.05	0.5	234.3
		N	10	10	10	10	10	10	10	9	10	10
3	m	Mean	6.84	3.21	3.63	0.90	110.9	86.3*	159.4	0.14	4.4	295.7
		S.D.	0.27	0.15	0.17	0.05	16.3	9.5	13.4	0.05	0.5	116.5
		N	9	9	9	9	9	9	9	8	9	9
4	m	Mean	6.95	3.24	3.71	0.86	116.2	89.1*	161.2*	0.12	4.6	288.5
		S.D.	0.23	0.13	0.14	0.05	26.7	12.7	19.7	0.04	0.7	147.3
		N	10	10	10	10	10	10	10	9	10	10

* - Significantly different from the control value, p ≤ 0.05

Nominal Dose (NVK004/RRT (b) (4) /RRT (b) (4) : Group 1- 0/0/0 µg/kg/day Group 2- 920/0/0 µg/kg/day
Group 3- 920/45.7/21.1 µg/kg/day Group 4- 920/91.3/42.2 µg/kg/day

Day 15 Relative to Start Date

Group	Sex		GLU mg/dL	BUN mg/dL	CRE2 mg/dL	NA mmol/L	K mmol/L	CL mmol/L	CA mg/dL	PHOS mg/dL	CHOL mg/dL	TRIG mg/dL
1	f	Mean	100.7	20.3	0.60	144.3	5.96	105.4	11.65	9.43	105.4	33.2
		S.D.	15.1	2.8	0.05	1.3	0.56	1.2	0.43	1.07	13.2	10.0
		N	10	10	10	10	10	10	10	10	10	10
2	f	Mean	93.7	30.6*	0.60	143.8	5.56	103.7*	11.74	9.96	115.2	36.0
		S.D.	7.8	4.1	0.07	1.7	0.35	1.5	0.42	1.68	14.8	10.5
		N	10	10	10	10	10	10	10	10	10	10
3	f	Mean	93.4	31.8*	0.62	143.0	5.83	103.7*	11.85	10.32	121.3	38.3
		S.D.	7.3	5.4	0.06	1.2	0.82	1.5	0.44	1.26	17.4	10.6
		N	10	10	10	10	10	10	10	10	10	10
4	f	Mean	98.1	30.1*	0.57	144.2	5.46	104.2	11.88	10.33	107.0	33.7
		S.D.	11.0	4.7	0.07	0.9	0.59	1.5	0.52	1.30	15.9	9.4
		N	10	10	10	10	10	10	10	10	10	10

* - Significantly different from the control value, p ≤ 0.05

Nominal Dose (NVK004/RRT (b) (4) /RRT (b) (4) : Group 1- 0/0/0 µg/kg/day Group 2- 920/0/0 µg/kg/day
Group 3- 920/45.7/21.1 µg/kg/day Group 4- 920/91.3/42.2 µg/kg/day

Day 15 Relative to Start Date

Group	Sex		TPROT g/dL	ALB g/dL	GLOB g/dL	A/G ratio	AST U/L	ALTi U/L	ALPi U/L	TBILI mg/dL	GGT U/L	CK U/L
1	f	Mean	6.89	3.45	3.44	1.02	108.0	59.1	105.3	0.16	4.0	188.4
		S.D.	0.31	0.14	0.23	0.08	16.2	7.5	18.4	0.05	0.7	62.9
		N	10	10	10	10	10	10	10	10	10	10
2	f	Mean	6.84	3.30	3.54	0.94	124.7	65.0	104.2	0.13	4.4	330.5
		S.D.	0.17	0.19	0.22	0.10	44.4	14.1	38.4	0.05	0.5	347.3
		N	10	10	10	10	10	10	10	10	10	10
3	f	Mean	7.18	3.53	3.65	0.95	117.5	73.8*	114.5	0.13	4.5	248.4
		S.D.	0.30	0.16	0.22	0.05	21.5	11.9	23.7	0.05	0.5	130.3
		N	10	10	10	10	10	10	10	10	10	10
4	f	Mean	6.96	3.32	3.64	0.91*	144.3	69.4	119.9	0.16	5.0*	364.4
		S.D.	0.28	0.28	0.25	0.11	58.1	8.6	34.9	0.05	0.7	325.1
		N	10	10	10	10	10	10	10	8	10	10

* - Significantly different from the control value, p ≤ 0.05

Nominal Dose (NVK004/RRT (b) (4) /RRT (b) (4) : Group 1- 0/0/0 µg/kg/day Group 2- 920/0/0 µg/kg/day
Group 3- 920/45.7/21.1 µg/kg/day Group 4- 920/91.3/42.2 µg/kg/day

Hematology

There were mild increases in mean WBC (up to 1.37x), ABNEUT (up to 2.24x) and ABMONO (up to 1.99x), relative to controls on day 15 in Group 4 (nominal 920/91.3/42.2 µg/kg/day) in both

sexes. These findings were consistent with inflammation; however, no microscopic correlates were seen, and the effect was not considered adverse as shown below.

Day 15 Relative to Start Date

Group	Sex		RBC M/µL	HGB g/dL	HCT %	MCV fL	MCH pg	MCHC g/dL	RDW %	RET %	ABRETi 10^9/L
1	m	Mean	8.468	15.34	50.21	59.30	18.11	30.51	12.23	2.91	246.68
		S.D.	0.283	0.32	1.64	1.66	0.48	0.44	0.43	0.48	44.25
		N	10	10	10	10	10	10	10	10	10
2	m	Mean	8.921	16.14	52.78	59.15	18.06	30.54	12.27	3.60	320.69*
		S.D.	0.462	0.84	3.29	1.29	0.25	0.39	0.37	0.50	52.84
		N	10	10	10	10	10	10	10	10	10
3	m	Mean	8.790	15.69	51.16	58.18	17.86	30.70	12.06	3.34	294.38
		S.D.	0.483	1.02	3.34	1.26	0.52	0.44	0.54	0.63	54.48
		N	9	9	9	9	9	9	9	9	9
4	m	Mean	8.934	15.95	52.68	59.00	17.87	30.33	12.46	3.73	333.33*
		S.D.	0.495	0.79	3.16	2.65	0.73	0.46	1.05	0.95	79.31
		N	10	10	10	10	10	10	10	10	10

* - Significantly different from the control value, p ≤ 0.05

Nominal Dose (NVK004/RRT (b) (4) RRT (b) (4): Group 1- 0/0/0 µg/kg/day Group 2- 920/0/0 µg/kg/day
Group 3- 920/45.7/21.1 µg/kg/day Group 4- 920/91.3/42.2 µg/kg/day

Day 15 Relative to Start Date

Group	Sex		PLT K/µL	MPV fL	WBC K/µL	ABNEUT K/µL	ABLYMP K/µL	ABMONO K/µL	ABSEOS K/µL	ABBAS K/µL
1	m	Mean	911.1	6.56	10.655	1.531	8.237	0.224	0.274	0.317
		S.D.	207.3	0.35	0.986	0.434	0.833	0.080	0.157	0.096
		N	10	10	10	10	10	10	10	10
2	m	Mean	841.0	6.60	13.038	1.895	10.237*	0.266	0.199	0.369
		S.D.	95.4	0.34	3.287	0.824	2.374	0.148	0.092	0.158
		N	10	10	10	10	10	10	10	10
3	m	Mean	887.9	6.57	12.903	2.373	9.551	0.349	0.193	0.358
		S.D.	140.3	0.49	2.052	1.582	1.537	0.190	0.105	0.170
		N	9	9	9	9	9	9	9	9
4	m	Mean	814.7	6.90	14.575*	3.426*	9.855	0.446*	0.285	0.468
		S.D.	128.5	0.33	2.036	2.458	1.093	0.142	0.131	0.118
		N	10	10	10	10	10	10	10	10

* - Significantly different from the control value, p ≤ 0.05

Nominal Dose (NVK004/RRT (b) (4) RRT (b) (4): Group 1- 0/0/0 µg/kg/day Group 2- 920/0/0 µg/kg/day
Group 3- 920/45.7/21.1 µg/kg/day Group 4- 920/91.3/42.2 µg/kg/day

Day 15 Relative to Start Date

Group	Sex		RBC M/µL	HGB g/dL	HCT %	MCV fL	MCH pg	MCHC g/dL	RDW %	RET %	ABRETi 10^9/L
1	f	Mean	8.009	14.84	48.24	60.22	18.52	30.75	11.40	3.04	241.49
		S.D.	0.209	0.56	1.62	1.14	0.39	0.46	0.31	0.51	39.07
		N	10	10	10	10	10	10	10	10	10
2	f	Mean	8.083	14.93	47.79	59.13	18.48	31.28	11.17	2.79	226.16
		S.D.	0.253	0.66	2.15	2.13	0.62	0.57	0.40	0.69	54.59
		N	10	10	10	10	10	10	10	10	10
3	f	Mean	8.285	15.31	49.44	59.68	18.48	30.96	11.70	3.51	283.12
		S.D.	0.595	1.05	3.19	1.21	0.26	0.44	1.00	1.81	117.20
		N	10	10	10	10	10	10	10	10	10
4	f	Mean	8.107	14.81	48.10	59.35	18.27	30.76	11.40	2.74	220.99
		S.D.	0.419	0.83	2.30	1.36	0.51	0.56	0.45	0.59	41.66
		N	10	10	10	10	10	10	10	10	10

Nominal Dose (NVK004/RRT (b) (4) RRT (b) (4): Group 1- 0/0/0 µg/kg/day Group 2- 920/0/0 µg/kg/day
Group 3- 920/45.7/21.1 µg/kg/day Group 4- 920/91.3/42.2 µg/kg/day

Day 15 Relative to Start Date

Group	Sex		PLT K/µL	MPV fL	WBC K/µL	ABNEUT K/µL	ABLYMP K/µL	ABMONO K/µL	ABSEOS K/µL	ABBAS K/µL
1	f	Mean	944.6	6.68	8.757	1.063	7.116	0.182	0.175	0.162
		S.D.	112.5	0.49	1.790	0.566	1.371	0.077	0.073	0.087
		N	10	10	10	10	10	10	10	10
2	f	Mean	851.6	6.90	9.892	1.629	7.581	0.272	0.135	0.191
		S.D.	115.9	0.52	3.155	1.307	2.142	0.145	0.072	0.086
		N	9	9	10	10	10	10	10	10
3	f	Mean	866.8	7.12	8.191	0.941	6.736	0.172	0.164	0.127
		S.D.	250.0	0.36	1.600	0.430	1.385	0.087	0.065	0.049
		N	10	10	10	10	10	10	10	10
4	f	Mean	822.2	7.32*	11.627*	2.033	8.790	0.329*	0.200	0.205
		S.D.	332.3	0.59	3.124	1.430	1.926	0.103	0.086	0.103
		N	10	10	10	10	10	10	10	10

* - Significantly different from the control value, p ≤ 0.05

Nominal Dose (NVK004/RRT (b) (4) RRT (b) (4): Group 1- 0/0/0 µg/kg/day Group 2- 920/0/0 µg/kg/day
Group 3- 920/45.7/21.1 µg/kg/day Group 4- 920/91.3/42.2 µg/kg/day

Coagulation

There were mild increases in mean FIB (up to 1.26x) relative to controls on day 15 in Group 4 (nominal 920/91.3/42.2 µg/kg/day) in both sexes. These values could indicate an inflammatory state as part of the acute-phase response and consistent with the WBC changes in group 4.

Day 15 Relative to Start Date

Group	Sex		PT sec	APTT sec	FIB mg/dL	Group	Sex		PT sec	APTT sec	FIB mg/dL
1	m	Mean	17.63	25.83	336.3	1	f	Mean	16.18	17.98	277.0
		S.D.	0.56	4.52	37.0			S.D.	0.90	2.70	33.8
		N	10	10	10			N	10	10	10
2	m	Mean	16.55*	26.02	375.4	2	f	Mean	15.88	15.96	342.8
		S.D.	1.46	6.52	38.1			S.D.	0.34	3.25	158.7
		N	10	10	10			N	9	9	9
3	m	Mean	17.53	16.77*	398.8	3	f	Mean	15.83	18.05	243.6
		S.D.	0.78	3.45	52.6			S.D.	0.73	3.51	62.5
		N	9	9	9			N	10	10	10
4	m	Mean	17.88	28.82	422.5*	4	f	Mean	16.52	16.16	316.7
		S.D.	0.83	8.57	119.9			S.D.	0.80	2.97	131.8
		N	10	10	10			N	9	9	9

* - Significantly different from the control value, p ≤ 0.05

Nominal Dose (NVK004/RRT (b) (4) RRT (b) (4)): Group 1- 0/0/0 µg/kg/day Group 2- 920/0/0 µg/kg/day Group 3- 920/45.7/21.1 µg/kg/day Group 4- 920/91.3/42.2 µg/kg/day

Macroscopic Findings

There were no test article-related macroscopic observations at terminal necropsy on Day 15. All macroscopic observations were considered not related to the administration of the NVK004 impurities because they occurred sporadically, were present at a similar incidence in control animals and test article exposed groups or were considered to represent incidental "background" findings that are seen commonly in SD rats of this age.

Organ Weights

Thyroid/parathyroids, ovaries/oviducts, and uterus/cervix were collected and weighed together. Kidney/BW and liver/BW ratios in males in group 3 and 4 were significantly different from the control values. Due to the fact that these changes were small in magnitude, affected only the absolute or relative weights and not both, and/or did not have macroscopic or microscopic correlates, organ weight changes at terminal necropsy, regardless of statistical significance, were considered unlikely to be related to the NVK004 impurities.

Microscopic Findings

Microscopic findings related to administration of the test articles were identified in the heart, stomach, small intestines (duodenum and ileum), large intestines (cecum and colon), kidneys, lungs, brain and aorta. These findings were generally present in animals administered NVK004 alone, NVK004 with 45.7 µg/kg/day RRT (b) (4) and 21.1 µg/kg/day RRT (b) (4), and NVK004 with 91.3 µg/kg/day RRT (b) (4) and 42.2 µg/kg/day RRT (b) (4). They were consistent with a hypertenstive effect of NVK004 on the cardiovascular system.

Cardiovascular system findings consisted of minimal or mild myocardial fibrosis in the heart; minimal degeneration/necrosis and/or minimal hypertrophy/hyperplasia of the tunica media of small arteries and arterioles in the heart, gastrointestinal tract and minimal periarterial mononuclear cell infiltrates in the gastrointestinal tract and brain. Other microscopic findings related to administration of the test articles consisted of minimal glomerulopathy affecting one or a few glomeruli in the kidneys; and an increased incidence and severity (minimal to moderate) of hemorrhage/ edema in the lungs, which was often accompanied by minimal crystalline alveolar pigment (interpreted as hemoglobin crystals) and minimal perivascular and alveolar hemosiderin

pigment. Hemosiderin pigment and crystalline alveolar pigment were consistent with prior episodes of hemorrhage. Effects of NVK004 on arteries and arterioles in the gastrointestinal tract were more frequently identified in males than females as shown below.

Text Table 2. Microscopic Findings Related to Administration of the Test Articles on Day 15

	Sex:	Male				Female			
	Group:	1	2	3	4	1	2	3	4
	Norepinephrine Dose (µg/kg/day):	0	920	920	920	0	920	920	920
	Nominal Impurity (b) (4) RRT (b) (4) Dose (µg/kg/day):	0	0	45.7	91.3	0	0	45.7	91.3
	Nominal Impurity RRT Dose (µg/kg/day):	0	0	21.1	42.2	0	0	21.1	42.2
Heart	Number Examined	10	10	9	10	10	10	10	10
Degeneration/ necrosis; media; artery	Minimal	0	0	0	0	0	2	0	0
Hypertrophy/ hyperplasia; media; artery	Minimal	0	8	9	7	0	10	9	10
Fibrosis; myocardium; multifocal	Minimal	0	8	7	6	0	4	3	7
	Mild	0	0	0	1	0	0	0	0
Stomach	Number Examined	10	10	9	10	10	10	10	10
Hypertrophy/ hyperplasia; media; artery	Minimal	0	2	2	1	0	1	1	1
Fibrosis; periarterial	Minimal	0	1	1	1	0	0	1	0
Infiltrate, mononuclear cell; periarterial	Minimal	0	1	1	1	0	1	0	0
Small Intestine, Duodenum	Number Examined	10	10	0	10	10	10	0	10
Hypertrophy/ hyperplasia; media; artery	Minimal	0	2	-	0	0	0	-	0
Infiltrate, mononuclear cell; periarterial	Minimal	0	1	-	0	0	0	-	0
Small Intestine, Ileum	Number Examined	10	10	0	10	10	10	0	10
Infiltrate, mononuclear cell; periarterial	Minimal	0	1	-	0	0	0	-	0
Large Intestine, Cecum	Number Examined	10	10	9	10	10	10	10	10
Hypertrophy/ hyperplasia; media; artery	Minimal	0	3	2	3	0	0	1	0
Fibrosis; periarterial	Minimal	0	2	1	2	0	0	1	0
Infiltrate, mononuclear cell; periarterial	Minimal	0	2	0	1	0	0	0	0
Large Intestine, Colon	Number Examined	10	10	9	10	10	10	10	10
Degeneration/ necrosis; media; artery	Minimal	0	0	0	1	0	0	0	0
Hypertrophy/ hyperplasia; media; artery	Minimal	0	3	5	4	0	0	1	0
Fibrosis; periarterial	Minimal	0	3	5	3	0	0	0	0
Infiltrate, mononuclear cell; periarterial	Minimal	0	4	3	3	0	1	0	0
Kidneys	Number Examined	10	10	9	10	10	10	10	10
Glomerulopathy	Minimal	0	1	3	0	0	2	1	1
Lungs	Number Examined	10	10	9	10	10	10	10	10
Degeneration/ necrosis; media; artery	Minimal	0	0	0	0	0	1	0	0
Hemorrhage/ edema	Minimal	6	4	2	5	6	5	4	5
	Mild	0	6	7	2	2	3	5	5
	Moderate	0	0	0	2	0	0	0	0
Pigment; hemosiderin; perivascular; alveolar	Minimal	0	5	7	3	0	2	4	2
Pigment, crystals; alveolar	Minimal	0	1	0	0	0	0	0	2

APPEARS THIS WAY IN ORIGINAL

Fibrosis; septal; multifocal	Mild	0	0	0	1	0	0	0	0
Sex:		Male				Female			
Group:		1	2	3	4	1	2	3	4
Norepinephrine Dose (µg/kg/day):		0	920	920	920	0	920	920	920
Nominal Impurity (b) (4) RRT (b) (4) Dose (µg/kg/day):		0	0	45.7	91.3	0	0	45.7	91.3
Nominal Impurity RRT Dose (µg/kg/day):		0	0	21.1	42.2	0	0	21.1	42.2
Brain	Number Examined	10	10	9	10	10	10	10	10
Degeneration/ necrosis; media; artery	Minimal	0	0	2	1	0	0	0	0
Infiltrate, mononuclear cell; periarterial	Minimal	0	0	1	1	0	0	0	0
Hypertrophy/ hyperplasia; media; artery	Minimal	0	0	1	0	0	0	0	0
Artery, Aorta	Number Examined	10	10	9	10	10	10	10	10
Necrosis; mural	Moderate	0	0	0	1	0	0	0	0
Hemorrhage	Mild	0	0	0	1	0	0	0	0
Inflammation, mixed	Mild	0	0	0	1	0	0	0	0

- = not applicable.

Adverse findings in the heart, arteries and arterioles (multiple organs), and kidneys were identified at similar incidence and severity in all three groups and were likely attributed to the effects of NVK004 on the cardiovascular system with presumptive NVK004-related hypertension. But some adverse findings in the lungs showed a higher incidence or severity in group 3 and 4, compared with group 2 (see below)

Lungs	Number Examined	10	10	9	10	10	10	10	10
Degeneration/ necrosis; media; artery	Minimal	0	0	0	0	0	1	0	0
Hemorrhage/ edema	Minimal	6	4	2	5	6	5	4	5
	Mild	0	6	7	2	2	3	5	5
	Moderate	0	0	0	2	0	0	0	0

Microscopic findings that were only present in animals administered NVK004 with 91.3 µg/kg/day RRT (b) (4) and 42.2 µg/kg/day RRT (b) (4) included the following:

- Degeneration/necrosis, inflammatory cell infiltrate and hypertrophy/hyperplasia of the media of arterial vessels in the brain in male animals.
- Mild multifocal septal fibrosis in the lungs of one animal (male 14835) and moderate mural necrosis of the ascending aorta of another animal (male 14840).

Fibrosis; septal; multifocal	Mild	0	0	0	1	0	0	0	0
Sex:		Male				Female			
Group:		1	2	3	4	1	2	3	4
Norepinephrine Dose (µg/kg/day):		0	920	920	920	0	920	920	920
Nominal Impurity (b) (4) RRT (b) (4) Dose (µg/kg/day):		0	0	45.7	91.3	0	0	45.7	91.3
Nominal Impurity RRT Dose (µg/kg/day):		0	0	21.1	42.2	0	0	21.1	42.2
Brain	Number Examined	10	10	9	10	10	10	10	10
Degeneration/ necrosis; media; artery	Minimal	0	0	2	1	0	0	0	0
Infiltrate, mononuclear cell; periarterial	Minimal	0	0	1	1	0	0	0	0
Hypertrophy/ hyperplasia; media; artery	Minimal	0	0	1	0	0	0	0	0
Artery, Aorta	Number Examined	10	10	9	10	10	10	10	10
Necrosis; mural	Moderate	0	0	0	1	0	0	0	0
Hemorrhage	Mild	0	0	0	1	0	0	0	0
Inflammation, mixed	Mild	0	0	0	1	0	0	0	0

- = not applicable.

The aortic necrosis was accompanied by mild hemorrhage and mild mixed inflammation in the periaortic soft tissue. While these findings were only identified in animals administered NVK004 with 91.3 µg/kg/day RRT (b) (4) and 42.2 µg/kg/day RRT (b) (4), they were consistent with a response to hypertension.

To understand whether the impurities have pharmacologic activities like NVK004 that could explain the above findings, an IR was sent to the applicant asking for this information:

(The applicant responded on 4/30/21)

Conclusion:
The data from 14 Day Repeat dose Toxicology study have shown that NVK004 alone or spiked with the impurities at (b) (4) or (b) (4) the maximum daily dose resulted in decreased body weight, mild increases in BUN in all treated groups.

NVK004 administration with impurities at (b) (4) the maximum daily dose, resulted in adverse effects on the heart, arteries and arterioles (multiple organs), lungs, and kidneys in all treated groups that were adverse and were attributed to the pharmacologic effects of NVK004 on the cardiovascular system with presumptive NVK004-related hypertension.

In view of the adverse findings on the heart, arteries and arterioles (multiple organs), lungs, and kidneys in all treated groups in NVK004 treated groups in 14 Day Repeat dose Toxicology Draft Study Report, a **Nonclinical IR** was submitted on April 26, 2021 as below:

Applicant Response on 4/30/2021
The Applicant has not conducted studies to assess the pharmacological activity of the two impurities (RRT (b) (4) and RRT (b) (4)) and no references have been found in the literature regarding their potential pharmacological activity. Nevertheless, based on the results of

(b) (4) Study No. 1602-20391, it cannot be excluded that either one or both impurities may be pharmacologically active.

The dose of each of the two impurities spiked at (b) (4) MDD and (b) (4) MDD, respectively, in (b) (4) Study No. 1602-20391 was set based on the proposed specifications of (b) (4)% for RRT (b) (4), and (b) (4)% for RRT (b) (4). It is important to note that to date (20 month long-term registration stability time point), levels of RRT (b) (4) have not exceeded (b) (4)% and RRT (b) (4) have not exceeded (b) (4)% in the 16 mcg/mL concentration, which is approximately (b) (4) fold lower than the proposed specifications, nor have they exceeded the qualification thresholds in the 32 mcg/mL and 64 mcg/mL concentrations.

Assuming some pharmacological activity that is like or mimics the effect of norepinephrine is present in the impurities, it is important to consider that the findings in the rats referenced by the Reviewer were generally characterized as minimal in the brain; mild for the aortic hemorrhage and fibrosis in the lung; and moderate for the mural necrosis. All the lesions reported are consistent with hypertensive responses following continuous IV administration in rats. On the other hand, since patients treated with the proposed product would be in an acute emergency hypotensive state, and not a prolonged hypertensive state as in the rats in the toxicology study, these lesions are unlikely to occur in the acute clinical setting. Further, hypotensive patients receiving norepinephrine in the acute emergency setting are monitored closely, so that even if transient hypertension were to occur with the administration of NVK004, it could be managed with dose adjustment or immediate discontinuation of the product."

Reviewer's Comment:

The degeneration/necrosis of the medial artery (2 observations at (b) (4) MDD and 1 observation at (b) (4) MDD) and hypertrophy/hyperplasia of the media artery (1 observation at (b) (4) MDD) with periarterial mononuclear cell infiltrates (1 observation at (b) (4) MDD) were observed in brains of 1 male rat (14841)treated at (b) (4) MDD (group 4) and 2 male rats (14816 and 14822)at (b) (4) MDD, however, no changes were seen in females.

Intergroup Comparison of Histopathology Observations for Aorta, Brain, and Lung

Terminal Necropsy: SD 15	MALES				FEMALES			
Group:	1	2	3	4	1	2	3	4
Number of Animals:	(10)	(10)	(9)	(10)	(10)	(10)	(10)	(10)
artery, aorta;								
Examined	(10)	(10)	(9)	(10)	(10)	(10)	(10)	(10)
Within Normal Limits	10	9	9	9	10	10	10	10
necrosis; mural	(0)	(0)	(0)	(1)	(0)	(0)	(0)	(0)
moderate	0	0	0	1	0	0	0	0
hemorrhage	(0)	(0)	(0)	(1)	(0)	(0)	(0)	(0)
mild	0	0	0	1	0	0	0	0
pigment; hemosiderin	(0)	(1)	(0)	(0)	(0)	(0)	(0)	(0)
minimal	0	1	0	0	0	0	0	0
inflammation, mixed	(0)	(0)	(0)	(1)	(0)	(0)	(0)	(0)
mild	0	0	0	1	0	0	0	0
brain;								
Examined	(10)	(10)	(9)	(10)	(10)	(10)	(10)	(10)
Within Normal Limits	10	10	7	9	10	10	10	10
degeneration/ necrosis; media; artery	(0)	(0)	(2)	(1)	(0)	(0)	(0)	(0)
minimal	0	0	2	1	0	0	0	0
infiltrate, mononuclear cell; periarterial	(0)	(0)	(1)	(1)	(0)	(0)	(0)	(0)
minimal	0	0	1	1	0	0	0	0
hypertrophy/ hyperplasia; media; artery	(0)	(0)	(1)	(0)	(0)	(0)	(0)	(0)
minimal	0	0	1	0	0	0	0	0

Nominal Dose (NVK004/RRT (b) (4) RRT (b) (4)): Group 1- 0/0/0 µg/kg/day Group 2- 920/0/0 µg/kg/day
Group 3- 920/45.7/21.1 µg/kg/day Group 4- 920/91.3/42.2 µg/kg/day

Terminal Necropsy: SD 15	MALES				FEMALES			
Group:	1	2	3	4	1	2	3	4
Number of Animals:	(10)	(10)	(9)	(10)	(10)	(10)	(10)	(10)
lung;								
Examined	(10)	(10)	(9)	(10)	(10)	(10)	(10)	(10)
Within Normal Limits	0	0	0	0	0	0	0	0
fibrosis; septal; multifocal	(0)	(0)	(0)	(1)	(0)	(0)	(0)	(0)
mild	0	0	0	1	0	0	0	0
hemorrhage	(1)	(0)	(0)	(0)	(1)	(0)	(0)	(0)
minimal	1	0	0	0	1	0	0	0
thrombosis	(1)	(0)	(0)	(0)	(0)	(0)	(1)	(0)
minimal	1	0	0	0	0	0	1	0
infiltrate, eosinophilic; perivascular	(10)	(10)	(8)	(9)	(10)	(10)	(10)	(10)
minimal	6	2	6	7	6	8	3	5
mild	4	8	2	2	4	2	7	5
pigment; hemosiderin; perivascular; alveolar	(0)	(5)	(7)	(3)	(0)	(2)	(4)	(2)
minimal	0	5	7	3	0	2	4	2
inflammation, mixed; alveolar/interstitial	(5)	(6)	(4)	(7)	(4)	(2)	(4)	(4)
minimal	4	4	4	5	4	2	3	4
mild	1	1	0	2	0	0	1	0
pigment, crystals; alveolar	(0)	(1)	(0)	(0)	(0)	(0)	(0)	(2)
minimal	0	1	0	0	0	0	0	2
increased macrophages; alveolar	(1)	(2)	(2)	(3)	(0)	(0)	(1)	(0)
minimal	1	2	2	2	0	0	1	0
mild	0	0	0	1	0	0	0	0
inflammation, granulomatous; alveolar/interstitial	(2)	(1)	(1)	(2)	(2)	(2)	(3)	(2)
minimal	2	1	1	2	2	2	3	2
metaplasia, osseous	(0)	(1)	(0)	(0)	(0)	(0)	(0)	(0)
minimal	0	1	0	0	0	0	0	0
degeneration/ necrosis; media; artery	(0)	(0)	(0)	(0)	(0)	(1)	(0)	(0)
minimal	0	0	0	0	0	1	0	0
hypertrophy/ hyperplasia; media; artery	(10)	(8)	(8)	(9)	(10)	(10)	(10)	(10)
minimal	5	4	5	4	6	7	5	7
mild	5	4	3	5	3	3	5	3
hemorrhage/edema	(6)	(10)	(9)	(9)	(8)	(8)	(9)	(10)
minimal	6	4	2	5	6	5	4	5
mild	0	6	7	2	2	3	5	5
moderate	0	0	0	2	0	0	0	0

Nominal Dose (NVK004/RRT (b) (4) RRT (b) (4): Group 1- 0/0/0 µg/kg/day Group 2- 920/0/0 µg/kg/day
Group 3- 920/45.7/21.1 µg/kg/day Group 4- 920/91.3/42.2 µg/kg/day

Source: NDA 214628 SN0012-CRL Study No. 1602-20391 Appendix 17: Pathology Report, Table 2-1B, Page 203

Moderate aortic artery mural necrosis with mild hemorrhage and a mixed inflammatory reaction were observed in one male rat (14840) in the (b) (4) MDD group (Group 4). Mild septal multifocal fibrosis in the lung were also seen in one male rat (14835) in the (b) (4) MDD group (Group 4).

Most histopathological changes were observed in NVK004 alone (group 2) as well asNVK004 spiked groups (group 3 and 4), at similar frequency or severity, suggesting the findings were due to NVK004. A few adverse findings mentioned above in the lung and brain occurred only in or with higher incidence or severity in group 3 and/or group 4, and these findings were generally consistent with a hypertensive response, suggesting that the spiked impurities might have pharmacologic activities that have contributed to the more pronounced hypertensive response. Since the indicated patient population is in a hypotensive stage and the dosing is individually titrated by the blood pressure response and tolerability, they are unlikely to experience prolonged hypertension as shown in the animal study.

Alternatively, the findings in the arteries in the lung and brain as well as in the aorta could result from direct toxicity of the impurities. There is not much pharmacology and/or toxicology information on these two impurities to exclude this possibility. In addition, the hypertensive response in group 2-4 is quite significant and could have masked some additional response by the impurities. If lower levels of NVK004 were given (as in the 28-day toxicity study) that produced lower background findings, the response by the impurities would be more easily differentiated and the lack of a response would have been more convincing.

In the applicant's response the 20 month long-term stability time point has shown that levels of RRT (b) (4) have not exceeded (b) (4)% and RRT (b) (4) have not exceeded (b) (4)% in the 16 mcg/mL concentration, which is approximately (b) (4)-fold lower than the proposed specifications and falls within the qualification threshold of 0.5% as per ICH Q3B (R2), nor have they exceeded the qualification thresholds in the 32 mcg/mL and 64 mcg/mL concentrations. This indicates that it is possible to control these impurities within the qualification threshold.

Recommendation

The NVK004 impurities were found to be non-genotoxic and non-hemolytic based on the totality of data from the battery of genotoxicity assays and the *in vitro* hemolysis study performed respectively.

14 Day Repeat dose Toxicology study has shown adverse findings in the aorta, the arteries in the brain and lungs as a result of the administration of two impurities RRT (b) (4) and RRT (b) (4) at (b) (4) or (b) (4) the maximum daily dose calculated from the proposed threshold of (b) (4)% and (b) (4)%, respectively.

These impurities belong to (b) (4) Some (b) (4) are known to cause neurotoxic effects (b) (4).

These two impurities are not degradation impurities and are not well characterized to exclude the possibility that they contributed to the additional toxicities observed in the groups spiked with the impurity mix in the 14-day repeat-dose toxicity study. We therefore recommend that a conservative approach be taken to control the levels of these impurities.

Based on the stability testing data at 20 months, the levels of RRT (b) (4) have not exceeded (b) (4)% and RRT (b) (4) have not exceeded (b) (4)% in the 16 mcg/mL concentration, which is approximately (b) (4) fold lower than the proposed specifications and falls within the qualification threshold of 0.5% as per ICH Q3B (R2). In addition, their levels have not exceeded the qualification thresholds in the 32 mcg/mL and 64 mcg/mL concentrations and these two impurities can be controlled within the qualification threshold of NMT 0.5% as per ICH-Q3B(R2).

From the pharmacology and toxicology perspective, we ***recommend*** that applicant revise the specification limit for these two impurities to be compliant with the qualification threshold set forth in ICH-Q3B(R2). Due to the uniqueness of these impurities (not being degradation impurities from the drug product. (b) (4) (b) (4)), we defer to OPQ for a final determination on the applicability of ICH-Q3A vs. ICH-Q3B in setting the impurity threshold.

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