Bayer CropScience

HPPD W336

ACUTE TOXICITY

BY INTRAVENOUS INJECTION IN MICE

DATA REQUIREMENTS
Based on the U.S. E.P.A. Health Effects Test Guidelines OPPTS 870.1100 adopted in 2002
and on the O.E.C.D. Test Guideline 425 adopted in 2001

REPORT OF STUDY SA 09154
Sponsor identification number: Lynx-PSI N° TX99L088

AUTHOR / STUDY DIRECTOR: J.B. RASCLE

TESTING FACILITY:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

SPONSOR:

Bayer AG
Bayer CropScience
Alfred Nobel Str. 50
40789 Monheim
Germany

STUDY COMPLETED ON: OCTOBER 13, 2009

STATEMENT OF NO DATA CONFIDENTIALITY CLAIM

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA § 10 (d) (1) (A), (B) or (C).

Company Name:

Company Agent:

Title:

Signature:

Date: _____

These data are the property of Bayer CropScience, and as such, are considered to be confidential for all purposes other than compliance with FIFRA § 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality which may exist under any other statute or in any other country.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study here reported was not performed in compliance with the principles of Good Laboratory Practice ("Bonnes Pratiques de Laboratoire") in that it was not subjected to specific Quality Assurance inspections. It was performed according to the standard operating procedures which were previously accepted and periodically inspected by the Quality Assurance Unit.

Author / Study Director:

Date: October 13, 2009

J.B. RASCLE

Sponsor Representative:

Date: October 13, 2009

A. CAPT

Study Submitter:

Date:

FLAGGING STATEMENTS

This page is reserved for flagging statements as may be required by U.S. E.P.A.

SIGNATURE

I, the undersigned, hereby declare that the work was performed under my supervision according to the procedures described and that this report provides a correct and faithful record of the results obtained.

There were no circumstances which affected the quality and integrity of the data.

Author / Study Director:

Date: October 13, 2009

J.B. RASCLE

STUDY PROFESSIONALS

The following professionals were involved in the conduct of this study:

STUDY DIRECTOR	:	J.B. RASCLE
LABORATORY ANIMAL RESOURCES	:	J.P. KOCWIN
TOXICOLOGY SUPERVISOR	:	D. SANTAMARIA
RESPONSIBLE TECHNICIAN	:	S. LEBAS
REPORT UNIT ASSISTANTS	:	M. VAGNER/P. ALMERAS

TABLE OF CONTENTS

SUMMARY

The objective was to assess the acute toxicity of the HPPD W336 protein administered via the intravenous route in female OF1 mice. The aprotinin protein was also tested in this study and acted as negative control.

Groups of 5 female OF1 mice were administered either with the HPPD W336 or the aprotinin protein in PBS buffer supplemented with 1µM $FeCl_3$ by intravenous injection at dose levels of 1 and 10 mg/kg body weight. All animals were observed for clinical signs daily for fifteen days whilst their body weights were measured weekly. At termination of the observation period, animals were subjected to a necropsy including macroscopic examination.

There were no mortalities and no treatment-related effects in female OF1 mice after an acute intravenous administration of the HPPD W336 at 1 or 10 mg/kg body weight.

In conclusion, the HPPD W336 was devoid of acute toxic potential up to 10 mg/kg (intravenous route).

INTRODUCTION

The objective was to assess the potential for acute toxicity of the HPPD W336 protein when administered intravenously at dose levels of 1 and 10 mg/kg body weight in the OF1 mouse. A negative control protein, aprotinin, was tested in parallel at similar dose levels.

The study protocol and its amendments are presented in Attachment 1.

The study time schedule was as follows:

Study initiation date *	May 19, 2009
Sponsor protocol approval date	May 19, 2009
Animal arrival date	May 20, 2009
Randomization date (Day -1)	May 26, 2009
Dosing date	May 27, 2009
Final sacrifice date	June 11, 2009
Experimental completion date	June 11, 2009

* Date of protocol approval by Study Director.

MATERIAL AND METHODS

1 - CONTROLS AND TEST SUBSTANCE FORMULATIONS

The certificates of analysis are presented in Attachment 2.

1.1 Test protein

The p-hydroxyphenylpyruvate dioxygenase (HPPD) from *Pseudomonas fluorescens*, carries a single glycine (G) to tryptophan (W) amino acid substitution at position 336 of the native enzyme, resulting in the HPPD W336 protein.
The test item HPPD W336 protein and the dilution buffer were supplied by BioAnalytics (Bayer BioScience NV, Zwijnaarde, Belgium).

Identification	:	HPPD W336 protein (produced in *Escherichia coli*)
Batch N°	:	LB150509
Description	:	In solution at 2.09 mg/mL in PBS supplemented with 1µM $FeCl_3$
Purity	:	>95 % on total protein
Storage	:	-74 +10°C*
Certified through	:	June, 2009

*: Storage condition at Sophia-Antipolis (SOP MTR00962).

The stock solution of the HPPD W336 protein (2.09 mg/mL) was diluted at the final concentration of 0.5 and 0.05 mg/mL in phosphate buffered saline (PBS) buffer (13.7 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) supplemented with 1µM $FeCl_3$.

1.2 Reference proteins

The reference protein aprotinin (1) is a serine protease inhibitor with a molecular weight of approximately 6 512 Da, (Sigma-Aldrich reference number A4529, batch number 058K7017). Chemical information on aprotinin was documented by Sigma-Aldrich and is presented in Attachment 2. This substance was stored in an air-tight, light-resistant container at approximately +4°C.

The aprotinin formulation was prepared by dissolving the protein in PBS buffer supplemented with 1µM $FeCl_3$ at the final concentration of 0.5 and 0.05 mg/mL.

The formulations were placed in air-tight glass bottles at room temperature and were used as quickly as practicable after preparation.

2 - ANIMALS, HOUSING, DIET AND WATER

2.1 Animals

The mouse was chosen because it is recommended by regulatory authorities as an appropriate test species to assess acute toxicity. The Crl:OF1 strain was used since sufficient background toxicity data exist to support interpretation of results. A total of 25 female Crl:OF1 mice were obtained from Charles River Laboratories, Saint Germain sur l'Arbresle, France. Animals were acclimatized to laboratory conditions for 7 days prior to treatment and were 8 weeks old at the treatment date.

a/Selection and randomization

All animals were examined daily for mortality and clinical signs during the acclimatization phase. The day before test substance administration, all animals were weighed. A computerized randomization procedure (Path/Tox system version 4.2.2), that ensured a similar body weight distribution among groups for each sex, was used to select animals for the study from the middle of the weight range of the available animals. Selected female animals were in a weight range from 26.2 to 30.6 g on the day of exposure to the test substance, i.e., within ±20% of the mean body weight on the day of randomization. Five female mice per group were selected for the study.

b/Identification

One day before treatment following randomization, animals were assigned permanent identification numbers within groups. Each animal was identified by a stainless steel ear tag bearing a unique animal number.

2.2 Housing

Mice were housed individually in suspended, stainless steel, wire-mesh cages. The temperature, humidity and lighting in the animal room were constantly monitored by an automatic system.

Environmental conditions:	**Target ranges:**	
	Temperature:	20-24 °C
	Humidity:	40-70 %
	Air changes:	10 to 15/hr
	Photoperiod:	12 hrs dark/ 12 hrs light (7 am- 7 pm)

There were no deviations from target specifications which could have compromised the study. Housing data are placed in the study file.

2.3 Diet and water

Certified rodent pelleted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animals Food and Engineering, Augy, France) was available *ad libitum*. Filtered and softened tap water from the municipal water supply was provided *ad libitum* using water bottles. Filters servicing the watering system were regularly changed and sterilization of the system was performed if considered necessary. Certificates of analysis were provided by the diet manufacturer and the supplier. Additionally, quality control analytical reports of the physicochemical properties and concentration of specified contaminants were periodically obtained from independent consultant analysts. These routine analyses of feed and water indicated that there was no contamination which could have affected the integrity and outcome of this study.

3 - EXPERIMENTAL DESIGN

Groups of 5 female mice were given a single intravenous injection of the test or control substance. The test or control proteins were administered in solution in PBS buffer supplemented with 1 µM $FeCl_3$ at dose levels of 1 or 10 mg/kg body weight, intravenously through the tail vein at a volume of 20 ml/kg (based on body weight on Study Day 1). The high dose was chosen in accordance with the Sponsor Representative , based on the preliminary safety assessment of the test substance, which lead to the conclusion with a high degree of certainty of the lack of harmful effects caused by the HPPD W336 protein after intravenous administration to mammals.

Details of group sizes and treatments:

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: aprotinin	1	5	TT1F3394 to 3398
2		10	5	TT2F3399 to 3403
3	HPPD W336	1	5	TT3F3404 to 3408
4		10	5	TT4F3409 to 3413

Clinical signs were recorded daily throughout the study. They were recorded approximately 30 minutes after dosing, periodically during the first 24 hours post-dosing and at least once each day thereafter. The nature, onset, severity, reversibility and duration of all clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health, such as blood or loose feces. In addition, animals were checked twice daily for mortality, except on weekends and public holidays when they were checked once daily.

4 - BODY WEIGHT

Each animal was weighed on pre-study Days 3, 7 and on study Days 1, 8 and 15.

5 - POST MORTEM PROCEDURES

At final sacrifice on Study Day 15, surviving animals were anesthetized by Isoflurane inhalation (Baxter, Maurepas, France), then exsanguinated under deep anesthesia before necropsy. Necropsy included macroscopic examination of abdominal and thoracic cavities, major organs and tissues. Significant macroscopic abnormalities were recorded.

6 - CALCULATIONS

Means and standard deviations were calculated for body weights and absolute body weight gains.

7 - DATA STORAGE

All raw data, supporting documents, as well as protocol, protocol amendments and final report are maintained in the document archive room. A test substance reference sample is retained in the area of the products storeroom defined for the archiving of test substances. All of the above will be archived for at least 10 years in the designated areas at:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

RESULTS

1 - MORTALITY (Tab. 1)

There was no mortality observed during the course of the study.

2 - CLINICAL SIGNS (Tab. 1)

There were no treatment-related clinical signs recorded throughout the treatment period in any group.

3 - BODY WEIGHT (Tab. 2, 3)

Throughout the study, mean body weight parameters were similar between groups of animals that received the HPPD W336 and the negative control protein.

4 - GROSS PATHOLOGY (Tab. 4)

No abnormality was observed in any animal in all groups.

CONCLUSION

There were no mortalities and no treatment-related effects in female OF1 mice after an acute intravenous administration of the HPPD W336 at 1 or 10 mg/kg body weight.

In conclusion, the HPPD W336 was devoid of acute toxic potential up to 10 mg/kg (intravenous route).

REFERENCE

1. Mossinger, H., Dietrich, W., 1998. Activation of hemostasis during cardiopulmonary bypass and pediatric aprotinin dosage. Ann. Thorac. Surg. 65, S45-50.

PROTOCOL DEVIATIONS

There was no protocol deviation during the course of the study.

Author / Study Director:

Date: October 13, 2009

J.B. RASCLE

ABBREVIATIONS

%	Percentage
°C	Degree Celsius
am	*Ante meridiem*
Da	Daltons
E.E.C.	European Economic Communities
E.P.A.	Environmental Protection Agency
g	Gram
GLP	Good Laboratory Practice
HPPD	p-hydroxyphenylpyruvate dioxygenase
M.A.F.F.	Ministry of Agriculture, Forestry and Fisheries
mg/kg	Milligram(s)/kilogram
ml/kg	Milliliter(s)/kilogram
mM	Millimolar
O.E.C.D.	Organization for Economic Cooperation and Development
pm	*Post meridiem*
QA	Quality Assurance
s	Scheduled sacrifice (death type in PathTox table)
SDEVS	Standard Deviation
Tab.	Table
U.S.	United States
USA	United States of America
v/v	Volume/volume
w/v	Weight/volume
#	Number

TABLES

TABLE 1 - **INDIVIDUAL CLINICAL SIGNS AND DEAD ANIMAL STATUS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: Aprotinin	1	5	TT1F3394 to 3398
2		10	5	TT2F3399 to 3403
3	HPPD W336	1	5	TT3F3404 to 3408
4		10	5	TT4F3409 to 3413

INDIVIDUAL CLINICAL SIGNS TABLE
Study number: SA 09154
DATES 26-May-09 TO 10-Jun-09
Study start date: 27-May-09

Acute Toxicity/Intravenous

DOSAGE LEVEL IN: mg/kg

ANIMAL		OBSERVATION	DAYS OBSERVED
TT1F3394	1.0	NORMAL THROUGHOUT INTERVAL	
TT1F3395	1.0	NORMAL THROUGHOUT INTERVAL	
TT1F3396	1.0	NORMAL THROUGHOUT INTERVAL	
TT1F3397	1.0	NORMAL THROUGHOUT INTERVAL	
TT1F3398	1.0	NORMAL THROUGHOUT INTERVAL	
TT2F3399	10.0	NORMAL THROUGHOUT INTERVAL	
TT2F3400	10.0	NORMAL THROUGHOUT INTERVAL	
TT2F3401	10.0	NORMAL THROUGHOUT INTERVAL	
TT2F3402	10.0	NORMAL THROUGHOUT INTERVAL	
TT2F3403	10.0	NORMAL THROUGHOUT INTERVAL	
TT3F3404	1.0	NORMAL THROUGHOUT INTERVAL	
TT3F3405	1.0	NORMAL THROUGHOUT INTERVAL	
TT3F3406	1.0	NORMAL THROUGHOUT INTERVAL	
TT3F3407	1.0	NORMAL THROUGHOUT INTERVAL	
TT3F3408	1.0	NORMAL THROUGHOUT INTERVAL	
TT4F3409	10.0	NORMAL THROUGHOUT INTERVAL	
TT4F3410	10.0	NORMAL THROUGHOUT INTERVAL	
TT4F3411	10.0	NORMAL THROUGHOUT INTERVAL	
TT4F3412	10.0	NORMAL THROUGHOUT INTERVAL	
TT4F3413	10.0	NORMAL THROUGHOUT INTERVAL	

NOTE: ! = Pre-study phase; " = Dosing phase 1

Dead Animal Status List for All Animals
Study number: SA 09154
Study start date: 27-May-09

Acute Toxicity/Intravenous

Animal Number	Grp	Sex	Study Phase	Date and Time Data was Entered	Oper. No.	Date of Death	Phase Day	Death Typ	Status	Term. Body Wt. (g)	Ow	Grs
TT1F3394	1	F	Dosing phase 1	11-Jun-09 09:00	26	11-Jun-09	16	s	Final phase sacrifice	25.5	-	C
TT1F3395	1	F	Dosing phase 1	11-Jun-09 09:01	26	11-Jun-09	16	s	Final phase sacrifice	30.7	-	C
TT1F3396	1	F	Dosing phase 1	11-Jun-09 09:01	26	11-Jun-09	16	s	Final phase sacrifice	26.2	-	C
TT1F3397	1	F	Dosing phase 1	11-Jun-09 09:02	26	11-Jun-09	16	s	Final phase sacrifice	25.9	-	C
TT1F3398	1	F	Dosing phase 1	11-Jun-09 09:03	26	11-Jun-09	16	s	Final phase sacrifice	26.0	-	C
TT2F3399	2	F	Dosing phase 1	11-Jun-09 09:03	26	11-Jun-09	16	s	Final phase sacrifice	26.5	-	C
TT2F3400	2	F	Dosing phase 1	11-Jun-09 09:04	26	11-Jun-09	16	s	Final phase sacrifice	26.7	-	C
TT2F3401	2	F	Dosing phase 1	11-Jun-09 09:07	26	11-Jun-09	16	s	Final phase sacrifice	24.6	-	C
TT2F3402	2	F	Dosing phase 1	11-Jun-09 09:08	26	11-Jun-09	16	s	Final phase sacrifice	27.2	-	C
TT2F3403	2	F	Dosing phase 1	11-Jun-09 09:08	26	11-Jun-09	16	s	Final phase sacrifice	26.7	-	C
TT3F3404	3	F	Dosing phase 1	11-Jun-09 09:08	26	11-Jun-09	16	s	Final phase sacrifice	24.1	-	C
TT3F3405	3	F	Dosing phase 1	11-Jun-09 09:09	26	11-Jun-09	16	s	Final phase sacrifice	25.9	-	C
TT3F3406	3	F	Dosing phase 1	11-Jun-09 09:09	26	11-Jun-09	16	s	Final phase sacrifice	26.2	-	C
TT3F3407	3	F	Dosing phase 1	11-Jun-09 09:10	26	11-Jun-09	16	s	Final phase sacrifice	26.3	-	C
TT3F3408	3	F	Dosing phase 1	11-Jun-09 09:10	26	11-Jun-09	16	s	Final phase sacrifice	26.6	-	C
TT4F3409	4	F	Dosing phase 1	11-Jun-09 09:10	26	11-Jun-09	16	s	Final phase sacrifice	26.5	-	C
TT4F3410	4	F	Dosing phase 1	11-Jun-09 09:11	26	11-Jun-09	16	s	Final phase sacrifice	24.4	-	C
TT4F3411	4	F	Dosing phase 1	11-Jun-09 09:11	26	11-Jun-09	16	s	Final phase sacrifice	29.1	-	C
TT4F3412	4	F	Dosing phase 1	11-Jun-09 09:11	26	11-Jun-09	16	s	Final phase sacrifice	24.6	-	C
TT4F3413	4	F	Dosing phase 1	11-Jun-09 09:12	26	11-Jun-09	16	s	Final phase sacrifice	24.4	-	C

Note: * = pretest animal no. P = partial data. C = complete data. - = no data.

TABLE 2 - **MEAN AND INDIVIDUAL BODY WEIGHTS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: Aprotinin	1	5	TT1F3394 to 3398
2		10	5	TT2F3399 to 3403
3	HPPD W336	1	5	TT3F3404 to 3408
4		10	5	TT4F3409 to 3413

Bayer CropScience
Center of Toxicology
Sophia-Antipolis
Mouse/OF 1

ANIMAL BODY WEIGHTS IN (G)
Study number: SA 09154
Study start date: 27-May-09

Printed: 25-Sep-09
Page: 1
Acute Toxicity/Intravenous

DOSAGE IN mg/kg			DAY OF PHASE		
ANIMAL		SEX	1	8	15
			FEMALE ANIMALS		
TT1F3394	1.0	F	29.1	31.1	30.5
TT1F3395			30.6	34.8	35.7
TT1F3396			28.0	30.2	30.8
TT1F3397			26.8	29.0	29.7
TT1F3398			27.8	30.3	30.4
		(n)	5	5	5
		MEANS	28.5	31.1	31.4
		SDEVS	1.4	2.2	2.4
TT2F3399	10.0	F	28.5	31.5	32.0
TT2F3400			27.1	30.4	31.2
TT2F3401			27.9	29.1	28.2
TT2F3402			27.1	28.8	31.5
TT2F3403			29.1	32.3	30.8
		(n)	5	5	5
		MEANS	27.9	30.4	30.7
		SDEVS	0.9	1.5	1.5
TT3F3404	1.0	F	26.2	26.8	28.2
TT3F3405			27.7	30.0	30.8
TT3F3406			29.9	31.1	31.5
TT3F3407			28.1	30.3	30.0
TT3F3408			27.7	28.7	30.2
		(n)	5	5	5
		MEANS	27.9	29.4	30.1
		SDEVS	1.3	1.7	1.2
TT4F3409	10.0	F	29.0	30.0	30.9
TT4F3410			24.9	26.8	28.2
TT4F3411			28.9	32.2	34.0
TT4F3412			26.9	28.5	29.0
TT4F3413			27.7	30.0	29.1
		(n)	5	5	5
		MEANS	27.5	29.5	30.2
		SDEVS	1.7	2.0	2.3

NOTE: DATA FOR Dosing phase 1

TABLE 3 - **MEAN AND INDIVIDUAL ABSOLUTE WEIGHT GAINS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: Aprotinin	1	5	TT1F3394 to 3398
2		10	5	TT2F3399 to 3403
3	HPPD W336	1	5	TT3F3404 to 3408
4		10	5	TT4F3409 to 3413

Bayer CropScience
Center of Toxicology
Sophia-Antipolis
Mouse/OF 1

ANIMAL ABSOLUTE WEIGHT GAINS IN (G)
Study number: SA 09154
ABSOLUTE WEIGHT GAINS REFERENCED TO Dosing phase 1 (DAY 1)
Study start date: 27-May-09

Printed: 14-Aug-09
Page: 1
Acute Toxicity/Intravenous

DOSAGE IN mg/kg / ANIMAL		SEX	DAY OF PHASE 8	DAY OF PHASE 15
			FEMALE ANIMALS	
TT1F3394	1.0	F	2.0	1.4
TT1F3395			4.2	5.1
TT1F3396			2.2	2.8
TT1F3397			2.2	2.9
TT1F3398			2.5	2.6
		(n)	5	5
		MEANS	2.6	3.0
TT2F3399	10.0	F	3.0	3.5
TT2F3400			3.3	4.1
TT2F3401			1.2	0.3
TT2F3402			1.7	4.4
TT2F3403			3.2	1.7
		(n)	5	5
		MEANS	2.5	2.8
TT3F3404	1.0	F	0.6	2.0
TT3F3405			2.3	3.1
TT3F3406			1.2	1.6
TT3F3407			2.2	1.9
TT3F3408			1.0	2.5
		(n)	5	5
		MEANS	1.5	2.2
TT4F3409	10.0	F	1.0	1.9
TT4F3410			1.9	3.3
TT4F3411			3.3	5.1
TT4F3412			1.6	2.1
TT4F3413			2.3	1.4
		(n)	5	5
		MEANS	2.0	2.8

NOTE: DATA FOR Dosing phase 1

TABLE 4 - **INDIVIDUAL GROSS PATHOLOGY FINDINGS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: Aprotinin	1	5	TT1F3394 to 3398
2		10	5	TT2F3399 to 3403
3	HPPD W336	1	5	TT3F3404 to 3408
4		10	5	TT4F3409 to 3413

Animal number	Sex	Group/ Subgroup	Date and time data was entered	Date data taken	Opr #	Tissue / Observation(s) Locator, Severity, Other, Distribution, Shape/Attachments, Texture
TT1F3394	F	1/1				GENERAL COMMENT
			11-Jun-09 11:25	11-Jun-09	83	All organs, no abnormality, , , , ,
TT1F3395	F	1/1				GENERAL COMMENT
			11-Jun-09 11:25	11-Jun-09	83	All organs, no abnormality, , , , ,
TT1F3396	F	1/1				GENERAL COMMENT
			11-Jun-09 11:25	11-Jun-09	83	All organs, no abnormality, , , , ,
TT1F3397	F	1/1				GENERAL COMMENT
			11-Jun-09 11:25	11-Jun-09	83	All organs, no abnormality, , , , ,
TT1F3398	F	1/1				GENERAL COMMENT
			11-Jun-09 11:26	11-Jun-09	83	All organs, no abnormality, , , , ,
TT2F3399	F	2/1				GENERAL COMMENT
			11-Jun-09 11:26	11-Jun-09	83	All organs, no abnormality, , , , ,
TT2F3400	F	2/1				GENERAL COMMENT
			11-Jun-09 11:26	11-Jun-09	83	All organs, no abnormality, , , , ,
TT2F3401	F	2/1				GENERAL COMMENT
			11-Jun-09 11:27	11-Jun-09	83	All organs, no abnormality, , , , ,
TT2F3402	F	2/1				GENERAL COMMENT
			11-Jun-09 11:27	11-Jun-09	83	All organs, no abnormality, , , , ,
TT2F3403	F	2/1				GENERAL COMMENT
			11-Jun-09 11:27	11-Jun-09	83	All organs, no abnormality, , , , ,
TT3F3404	F	3/1				GENERAL COMMENT
			11-Jun-09 11:28	11-Jun-09	83	All organs, no abnormality, , , , ,
TT3F3405	F	3/1				GENERAL COMMENT
			11-Jun-09 11:28	11-Jun-09	83	All organs, no abnormality, , , , ,
TT3F3406	F	3/1				GENERAL COMMENT
			11-Jun-09 11:29	11-Jun-09	83	All organs, no abnormality, , , , ,
TT3F3407	F	3/1				GENERAL COMMENT
			11-Jun-09 11:29	11-Jun-09	83	All organs, no abnormality, , , , ,

Animal number	Sex	Group/ Subgroup	Date and time data was entered	Date data taken	Opr #	Tissue / Observation(s) Locator, Severity, Other, Distribution, Shape/Attachments, Texture
TT3F3408	F	3/1				GENERAL COMMENT
			11-Jun-09 11:29	11-Jun-09	83	All organs, no abnormality, , , , ,
TT4F3409	F	4/1				GENERAL COMMENT
			11-Jun-09 11:30	11-Jun-09	83	All organs, no abnormality, , , , ,
TT4F3410	F	4/1				GENERAL COMMENT
			11-Jun-09 11:30	11-Jun-09	83	All organs, no abnormality, , , , ,
TT4F3411	F	4/1				GENERAL COMMENT
			11-Jun-09 11:30	11-Jun-09	83	All organs, no abnormality, , , , ,
TT4F3412	F	4/1				GENERAL COMMENT
			11-Jun-09 11:30	11-Jun-09	83	All organs, no abnormality, , , , ,
TT4F3413	F	4/1				GENERAL COMMENT
			11-Jun-09 11:31	11-Jun-09	83	All organs, no abnormality, , , , ,

ATTACHMENTS

ATTACHMENT 1 - **PROTOCOL AND AMENDMENTS**

HPPD W336
ACUTE TOXICITY BY INTRAVENOUS INJECTION IN MICE

TESTING FACILITY:
Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

SPONSOR:
Bayer AG
Bayer CropScience
Alfred Nobel Str. 50
40789 Monheim
Germany

1 GENERAL

1.1 PURPOSE OF STUDY

The objective of this study is to investigate the acute toxicity of the HPPD W336 protein after intravenous injection into mice.

1.2 GOOD LABORATORY PRACTICE COMPLIANCE

This study will not be subjected to specific Quality Assurance inspections. However, standardized, routine operating methods similar to those used on this type of study are periodically inspected.

2 STUDY PERSONNEL

2.1 STUDY DIRECTOR:

Date: May 19, 2009 J.B. RASCLE

2.2 SPONSOR REPRESENTATIVE:

Date: 19 MAY 2009 A. CAPT

2.3 OTHER STUDY PERSONNEL

Responsibility		Name
In-life Supervisor	:	D. SANTAMARIA
Responsible Technician	:	S. LEBAS

Other study personnel will be identified as appropriate in the study file.

3 PROPOSED DATES

Arrival of animals	:	May 20, 2009	
Experimental starting date	:	May 20, 2009	
Randomization	:	May 26, 2009	
Start of treatment	:	May 27, 2009	
Final sacrifice date	:	June 11, 2009	
Experimental completion date	:	June 11, 2009	(estimated)

4 OVERVIEW OF STUDY DESIGN

Groups of 5 female mice will be treated at two dose levels (1 and 10 mg/kg/body weight) with aprotinin protein (negative control protein) or with HPPD W336 protein in order to identify any acute toxic effects of the test protein.

Animals will be observed for clinical signs daily for 15 days. Body weight will be recorded at weekly intervals and on the day of necropsy. All mice, including those found dead or killed for humane reasons during the study, will be subjected to macroscopic observations and, when deemed appropriate by the Study Director or the Sponsor Representative, tissues will be retained for possible microscopic examinations.

5 TEST SYSTEM

5.1 SPECIES SPECIFICATIONS

5.1.1 Species and strain

Species: Mouse

Sex: Female

Strain: Crl:OF1

Body weight at study start: 20-30 g

5.1.2 Animal supplier

Charles River France Laboratories (Saint Germain sur l'Arbresle, France).

5.1.3 Reason for selection of species

The mouse has been chosen because of its acceptance by Regulatory Authorities as a test species to assess acute toxicity.

The OF1 strain has been used extensively in toxicity evaluation studies, hence sufficient background data exist to support interpretation of results.

5.1.4 Age range and number

Twenty five female mice will be ordered. Animals will be 8 weeks of age at the start of exposure to the test substance.

5.1.5 Acclimatization phase and randomization

The duration of the acclimatization phase will be at least 7 days.

Animals will be checked twice daily for moribundity and mortality except on weekends and public holidays when they will be checked once daily.

All animals will be weighed at least weekly during the acclimatization phase.

The acceptable body weight range will be ±20% of the mean body weight on the day of randomization.

Any animal deemed unsuitable for the study based on weight or clinical signs will not be used in the study.

From the remaining animals, five mice will be allocated to the (dosage) groups by using a computerized randomization procedure that ensures a similar body weight distribution within this group.

5.1.6 Identification

At the time of randomization each animal will be identified by a stainless steel ear tag bearing a unique animal number.

5.2 DIET INFORMATION

5.2.1 Food

Certified rodent pelleted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animals Food and Engineering, Augy, France) will be available *ad libitum*.

Food will be stored in an identified room controlled for temperature and humidity. Diet will be used only until date of expiry.

Animals will be diet fasted overnight prior to final sacrifice.

5.2.2 Water

Filtered and softened tap water from the municipal water supply will be provided *ad libitum* using automatic watering system. Filters servicing the watering system are changed regularly and sterilization of the system will be performed if considered necessary.

5.2.3 Analyses

Analytical data will be provided by the manufacturer for each batch of diet including the size of pellets and concentration of nutritional components, selected heavy metals, pesticides, mycotoxins, microorganisms and nitroso compounds. Batches of diet will be only released for use after confirmation they meet specification.

Certificates of water analysis will be provided by the "Laboratoire Municipal d'Hygiène de la Ville de Nice" (France) and "Institut d'Hygiène Alimentaire de Longjumeau" (France).

5.2.4 Records

Records of certificates of food and water analyses will be retained in the archives.

5.3 ENVIRONMENTAL CONDITIONS

5.3.1 Room

Animal room number: L8

The animal room is within a barrier maintained unit with restricted entry.

5.3.2 Housing

Animals will be housed individually in suspended, stainless steel, wire mesh cages. The cage of each animal will be identified by a card bearing a unique identification number.

5.3.3 General environment

Temperature, humidity and ventilation:
Laboratory conditions will be controlled to ensure a temperature of 20°C - 24°C and a relative humidity of 40% - 70% with a target of 10 to 15 air changes per hour.

Lighting:
12-hour light/dark cycles will be provided by automatically controlled fluorescent-tube lighting (7am - 7pm).

Monitoring:
The temperature, humidity and lighting in the animal room are constantly monitored by an automatic system. The ventilation system in the animal room is maintained to ensure adequate ventilation, with the performance of the system regularly checked. Records of all deviations from specifications will be placed in the study file.

6 CONTROL AND TEST SUBSTANCES

6.1 CONTROL SUBSTANCE

The negative control protein aprotinin (reference A4529 Sigma-Aldrich, Saint Quentin Fallavier, France) will be resuspended in Phosphate-Buffered Saline (PBS) supplemented with 1µM $FeCl_3$.

6.2 TEST SUBSTANCE CHARACTERISTICS

6.2.1 Identification of test substance

The p-hydroxyphenylpyruvate dioxygenase (HPPD) from *Pseudomonas fluorescens*, carries a single glycine (G) to tryptophan (W) amino acid substitution at position 336 of the native enzyme, resulting in the HPPD W336 protein.

Test substance	:	HPPD W336
Batch number	:	Will be defined by amendment
Purity	:	Will be defined by amendment
Concentration	:	1 mg/mL
Certified through	:	Will be defined by amendment

Full details of the test substance description including its chemical structure and physical appearance will be included in the final report.

The test substance will be supplied by the Sponsor (Bayer BioScience NV, Gent, Belgium). The storage of a retention sample is under the responsibility of the Sponsor.

6.2.2 Storage

The test substance will be stored frozen in an air-tight, light-resistant container in an ultrafreezer or according to the conditions described in the test substance specifications when available. The storage stability is certified through the period of usage in the current study

6.2.3 Safety handling and requirements

Information on the appropriate safety precautions when handling the test substance will be given by the supplier or the Sponsor Representative.

In the absence of information on the potential toxic effects of the control and test substances, safety precautions will be applied according to the relevant standard operating procedures.

6.2.4 Analyses

The confirmation of the identity, purity and activity of the controls and the test substances will be provided by the supplier and the Sponsor Representative respectively. The certificate of analysis of the test substance will be included in the final study report.

6.3 TEST SUBSTANCE FORMULATION

6.3.1 Preparation, shipment and storage conditions

The test substance will be received from the Sponsor as a formulated solution. This formulated solution is under the responsibility of the Sponsor.

The formulation will be shipped under frozen conditions and will be stored at -20°C or colder at arrival. On the day of dosing, it will be thawed and gently homogenized before administration. The unused residue of the formulation will be stored at -20°C or colder.

6.3.2 Analyses

Stability of the test protein will be analyzed by BioAnalytics (Bayer BioScience NV, Gent, Belgium) at the same concentration and in the same buffer than in the present study, for a period that covers the duration of the treatment. Concentration of the test and reference proteins will not be analyzed. The homogeneity of the formulations for the reference and the test item will be checked by a visual inspection.

7 ROUTE OF ADMINISTRATION AND TREATMENT GROUPS

7.1 CHOICE OF DOSES

The doses of 1 and 10 mg HPPD W336/kg body weight were selected in accordance with the Sponsor Representative.

This choice was based on the preliminary safety assessment of the test substance, which led to the conclusion with a high degree of certainty of the lack of harmful effects caused by the HPPD W336 after intravenous administration to mammals.

Aprotinin is a serin protease inhibitor, non toxic when administered by intravenous route up to 10 mg/kg in mice. This dose is above the dose levels used in high dose efficacy studies with aprotinin (4.2 mg/kg body weight) in humans (1).

7.2 CHOICE OF ROUTE OF ADMINISTRATION

The intravenous route was selected to ensure systemic exposure to the test substance and was based on previous information obtained in acute mouse studies. Historically, the intravenous route of exposure has been used to investigate the toxicity of many proteins.

7.3 NUMBER OF ANIMALS

Five female mice per group will be administered with the appropriate concentrations of control or the test substances.

7.4 CONDITIONS OF ADMINISTRATION

All groups used in the study will receive the appropriate concentrations of control or test substance in vehicle at a constant volume of 10 ml/kg.

7.5 DOSES

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: Aprotinin	1	5	TT1F3394 to 3398
2		10	5	TT2F3399 to 3403
3	HPPD W336	1	5	TT3F3404 to 3408
4		10	5	TT4F3409 to 3413

7.6 ANIMAL WELFARE

During the study the care and use of animals will be conducted in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, n°86/609/CEE du 24 Novembre 1986".

8 LABORATORY DETERMINATIONS AND SCHEDULES

All animal data will be recorded using a dedicated computer system (Path/Tox System version 4.2.2, protocol number 0948).

8.1 CLINICAL EXAMINATION

8.1.1 Clinical signs and mortality

Clinical signs will be recorded individually starting on Day 1, at least once during the first 30 minutes after dosing, periodically during the first 24 hours post-dosing, and every day thereafter through Day 15. Additional observations will be necessary if the animals continue to display signs of toxicity. The nature, onset, severity, reversibility and duration of clinical signs will be recorded.

During the acclimatization phase and throughout the study, animals will be checked twice daily for moribundity and mortality (once daily on weekends and public holidays). Any animal suffering from severe distress, in a moribund condition or considered unlikely to survive will be humanely killed, and will be considered in the interpretation of the test results in the same way as animals that died on test.

8.1.2 Physical examination

Detailed physical examination will be performed at least weekly during the treatment period (commencing on Study Day 1).

8.1.3 Body weight

Body weights will be measured on Study Days 1 (shortly before the test substance is administered), 8 and 15.

8.2 POST MORTEM EXAMINATION

8.2.1 Necropsy procedures

Animals found dead:

Any animal found dead during the study will be necropsied as soon as possible but within 24 hours of the time of discovery. If necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen).

Scheduled sacrifice and moribund animals:

Animals surviving to the end of the study and animals sent to necropsy for humane reasons will be deeply anesthetized by Isoflurane (Baxter, Maurepas, France) inhalation, then exsanguinated before necropsy. All animals will be diet fasted prior to scheduled sacrifices.

8.2.2 Necropsy

The necropsy of animals will include the macroscopic examination of the external surface, all orifices and all major body cavities and organs.

Significant macroscopic findings will be recorded. Tissues may be sampled at the discretion of the Study Director, the Sponsor Representative or the Study Pathologist.

8.2.3 Microscopic evaluation

Microscopic examination of organs showing evidence of gross pathology in animals surviving at least 24 hours may be analyzed.

9 CALCULATIONS

For body weights, means and standard deviations will be calculated when possible.

10 REPORTING

10.1 INTERIM REPORTS

Any unexpected findings during the course of the study will be reported to the Sponsor Representative.

10.2 FINAL REPORT

A copy of the draft report will be submitted to the Sponsor Representative and the Quality Assurance Unit for review. With the exception of the dated signature of scientists and other professional personnel, the draft report will contain all information and data to be included into the final report.

11 ARCHIVING

All raw data, supporting documents, as well as protocol, protocol amendments, protocol deviations and the final report will be maintained in the archive room. An aliquot of the test substance reference samples and will be maintained in the archived sample room.

All of the above will be saved for at least ten years in the designated areas at:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

12 GENERAL REFERENCES

MOSSINGER, H., DIETRICH, W., 1998: Activation of hemostasis during cardiopulmonary bypass and pediatric aprotinin dosage. *Ann. Thorac. Surg.* 65, S45-50.

PROTOCOL AMENDMENT

Protocol **SA 09154**

HPPD W336
ACUTE TOXICITY BY INTRAVENOUS INJECTION
IN MICE

Protocol amendment: N°1

Reason: Modification of the conditions of administration

All groups used in the study will receive the appropriate concentrations of control or test substance in vehicle at a constant volume of 20 ml/kg, and not 10 ml/kg as originally stated in section 7.4 of the study protocol.

Study Director:

Date: May 27 2009

JB. RASCLE

PROTOCOL AMENDMENT

Protocol **SA 09154**

HPPD W336
ACUTE TOXICITY BY INTRAVENOUS INJECTION
IN MICE

Protocol amendment: N°2

Reason: Informations concerning the Test substance characteristics

The full details of the test substance used in the present study are given below:

Identification	: HPPD W336 protein (produced in *Escherichia coli*)
Batch N°	: LB150509
Description	: In solution at 2.09 mg/mL in PBS supplemented with 1µM $FeCl_3$
Purity	: >95 % on total protein
Storage	: -74 +10°C*
Certified through	: June, 2009

*: Storage condition at Sophia-Antipolis (SOP MTR00962)

Study Director:

Date: August 26, 2009

JB. RASCLE

ATTACHMENT 2 - **CERTIFICATES OF ANALYSIS**

Bayer CropScience

BioScience

CERTIFICATE OF ANALYSIS

Bayer BioScience N.V.
BioAnalytics
Technologiepark 38
B-9052 Zwijnaarde
Belgium

Origin of the Certified Material
Bayer BioScience N.V.
BioAnalytics
Technologiepark 38
B-9052 Zwijnaarde, Belgium
Tel : +32 (0)9 243 0411
Fax : +32 (0)9 224 0694

General Protein Information

- Product name : HPPD W336 protein
- Batch number : **LB180509**
- Produced by Bayer Bioscience N.V., Zwijnaarde, Belgium
- Molecular weight : deduced MW of 40.3 kDa is confirmed
- Buffer : PBS + 1 µM $FeCl_3$
- OD_{280} concentration: 2.09 mg/mL
- SDS-PAGE : - Reduced conditions
  - 10 % acrylamide gel
- Purity : > 95 % on total protein
  OD_{280}/OD_{260} ratio : 1.72
  (Attachment1)
- Western Blot :
  - Blotted on ProBlott-membrane in CAPS buffer
  - Blocking in Startingblock™ (PBS) blocking buffer (Thermo Scientific)
  - Primary antibody : Monoclonal anti-HPPD (Stine Biotechnology A36440: 1/1400)
  - Secondary antibody : Rabbit anti-mouse AP (Sigma A1902: 1/1000)

  (Attachment 2)

Bayer CropScience

BioScience

- Endotoxin : < 100 EU/ml
- Activity : Activ protein
- Stability : 4 hours after thawing
- Storage : at -70°C till use
  If thawed keep on ice
- Attachments : 2
- Reference : notebook 01380

Responsible Scientist

26/05/2009

Ann De Wulf, date

MC Manager

26/05/09

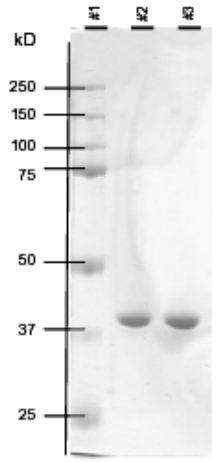
Jean-Marc Ferullo, date

2

Bayer CropScience

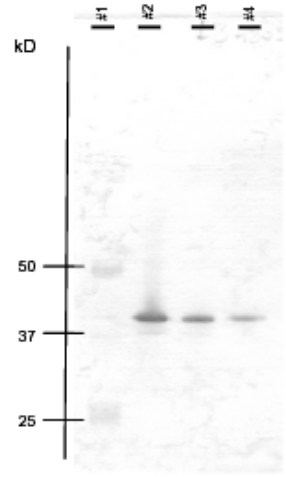
BioScience

Attachment 1: SDS-PAGE on 10% BT gel in MOPS buffer



Lane 1 : Molecular weight marker BioRad
Lane 2 : 4.2 µg LB180509
Lane 3 : 4.2 µg LB180509

Attachment 2: Western Blot



Lane 1 : Molecular weight marker BioRad
Lane 2 : 450 ng LB180509
Lane 3 : 150 ng LB180509
Lane 4 : 45 ng LB180509

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Aprotinin from bovine lung, lyophilized powder, 3-7 TIU/mg solid
Product Number	A4529
Product Brand	SIGMA
CAS Number	9087-70-1
Molecular Formula	$C_{284}H_{432}N_{84}O_{79}S_7$
Molecular Weight	6511.44
Storage Temp	2-8°C

TEST	SPECIFICATION	LOT 058K7017 RESULTS
APPEARANCE	REPORT RESULT	WHITE LYOPHILIZED POWDER
ACTIVITY	3 TO 7 TIU/MG SOLID	4.2 TIU/MG SOLID
UNIT DEFINITION	ONE TRYPSIN INHIBITOR UNIT (TIU) WILL DECREASE THE ACTIVITY OF 2TRYPSIN UNITS BY 50% WHERE ONE TRYPSIN UNIT WILL HYDROLYZE 1.0MICROMOLE OF N-ALPHA-BENZOYL-DL-ARGININE P-NITROANILIDE (BAPNA) PER MINUTE AT PH7.8 AT 25DEGC.	
RECOMMENDED RETEST	2 YEARS	MAY 2010
QC RELEASE DATE		MAY 2008

Rodney Burbach

Rodney Burbach, Manager
Quality Control
St. Louis, Missouri USA

FINAL REPORT AMENDMENT

There is no final report amendment at this time.

This page has been left blank intentionally