Study title: **EMD 415722 (Batch 2070) –Bacterial Mutagenicity Assay, *Salmonella Typhimurium* and *Escherichia Coli***

Key findings: EMD 415722 (hydroxocobalamin) at doses up to 5000 µg/plate was not mutagenic to strains of *S. typhimurium* and *E. coli* in the absence or presence of S9 mix.

Study no.: T15950
Vol. 11, Tab 4.2.3.3.1.2, T15950
Conducting laboratory and location: Institute of Toxicology, Merck KGaA, 64271 Darmstadt, Germany
Date of study initiation: Dec 1, 2004
GLP compliance: yes
QA reports: yes
Drug, lot #, and % purity:
EMD 415722 (hydroxocobalamin), Batch 2070, Purity 96.1%
Vehicle: physiological saline

Methods

The methods were the same as described for Study T15570.

Strains/species/cell line:
Salmonella typhimurium TA98, TA100, TA102, TA1535, TA1537
Escherichia coli WP2 *uvrA*

Doses used in definitive study: 50.0, 158, 500, 1580 and 5000 µg/plate

Basis of dose selection: see Methods for Study T15570.

Negative controls: physiological saline

Positive controls:

Strain	-S9		+S9	
	Compound	Dose (µg/plate)	Compound	Dose (µg/plate)
TA98	Daunomycin	4	2-aminoantracene	2
TA100	N-ethyl-N'-nitro-N-nitrosoguanidine	5	2-aminoantracene	2
TA102	cumene hydroperoxide	200	benzo[a]pyrene	10
TA1535	N-ethyl-N'-nitro-N-nitrosoguanidine	10	2-aminoantracene	2
TA1537	9-aminoacridine	50	2-aminoantracene	10
WP2 *uvrA*	N-ethyl-N'-nitro-N-nitrosoguanidine	5	2-aminoantracene	10

Incubation and sampling times: see Methods for Study T15570.

Results

Study validity: see Methods for Study T15570

Study outcome:

There was no precipitation of EMD 415722 on the agar plates. There was no clear toxicity to the bacteria, except for weak toxicity in the first experiment with TA1535 at the highest concentration only. Each treatment with the positive controls led to a clear increase in revertant colonies, thus, showing the expected reversion properties of all strains and good metabolic activity of the S9 mix used. Negative and positive controls were within their expected ranges.

EMD 415722 showed no increase in the number of revertants of any bacterial strain with and without the addition of rat liver S9 mix (series 2 Table below, reproduced from the sponsor's submission). Thus, EMD 415722 was not mutagenic in this assay.

TABLE 3 / Series No.: 2

EMD 415722: Summary of the Mean Number of Revertant Colonies T15950

Test Material	Concentration [µg/plate]	+/- S9-Mix	Mean revertant colonies / plate TA 98	TA 100	TA 102
Solvent control		-	19	149	252
EMD 415722	50	-	17	158	269
	158	-	21	162	279
	500	-	20	171	315
	1580	-	24	168	298
	5000	-	27	164	259
Solvent control		+	27	200	266
EMD 415722	50	+	32	213	282
	158	+	34	209	299
	500	+	36	200	304
	1580	+	34	221	311
	5000	+	34	224	275
Positive controls	Name		DAUN	ENNG	CUM
	Conc.[µg/plate]	-	4	5	200
	Revert./plate		894	682	1202
	Name		2-AA	2-AA	B(a)p
	Conc.[µg/plate]	+	2	2	10
	Revert./plate		139	479	1002

Test Material	Concentration [µg/plate]	+/- S9-Mix	Mean revertant colonies / plate TA 1535	TA 1537	WP2 uvrA
Solvent control		-	20	5	36
EMD 415722	50	-	19	8	51
	158	-	19	5	48
	500	-	24	7	45
	1580	-	22	7	41
	5000	-	27	9	54
Solvent control		+	25	8	41
EMD 415722	50	+	22	12	51
	158	+	22	12	52
	500	+	22	15	45
	1580	+	20	11	54
	5000	+	20	15	53
Positive controls	Name		ENNG	9-AA	ENNG
	Conc.[µg/plate]	-	10	50	5
	Revert./plate		378	630	1040
	Name		2-AA	2-AA	2-AA
	Conc.[µg/plate]	+	2	10	10
	Revert./plate		123	200	127

DAUN Daunomycin
CUM Cumene hydroperoxide
B(a)p Benzo(a)pyrene
ENNG N-Ethyl-N'-nitro-N-nitroso-guanidine
2-AA 2-Aminoanthracene
9-AA 9-Aminoacridine

Study title: **EMD 415722 (Batch 9337) – Bacterial Mutagenicity Assay, *Salmonella Typhimurium* and *Escherichia Coli***

Key findings: EMD 415722 (hydroxocobalamin) at doses up to 5000 µg/plate was not mutagenic to strains of *S. typhimurium* and *E. coli* in the absence or presence of S9 mix.

NOTE: This study was completed with this batch of drug substance since this batch contains a higher level of impurities in the drug substance. According to the sponsor, the impurity levels in this batch are as follows:

Batch	**9337**
Manufacturing Date	Aug 30, 2000
Release Date (stated in study)	Jan 29, 2001
used for Stability testing	**stored at 25°C/60% RH for 36 months**
Analysis Date	March 31, 2004 (Retest with the proposed HPLC method) this is 2 months after the end of 36 months of stability testing (43 months after manufactured)
[redacted]	[redacted]
Any other unspecified impurity	Max= [redacted]
Total impurity content	[redacted]

b(4)

Study no.: T15917
Vol. 12, Tab 4.2.3.7.6.3 T15917
Conducting laboratory and location: Institute of Toxicology Merck KGaA, 64271 Darmstadt, Germany
Date of study initiation: Nov 4, 2004
GLP compliance: yes
QA reports: yes
Drug, lot #, and % purity:
EMD 415722 (hydroxocobalamin), Batch 9337, Purity 92.4%
Vehicle: physiological saline

Methods

The methods were the same as described for Study T15570.

Strains/species/cell line:
Salmonella typhimurium TA98, TA100, TA102, TA1535, TA1537
Escherichia coli WP2 *uvrA*

Doses used in definitive study: 50.0, 158, 500, 1580 and 5000 µg/plate

Basis of dose selection: see Methods for Study T15570.

Negative controls: physiological saline

Positive controls:

Strain	-S9		+S9	
	Compound	Dose (µg/plate)	Compound	Dose (µg/plate)
TA98	Daunomycin	4	2-aminoantracene	2
TA100	N-ethyl-N'-nitro-N-nitrosoguanidine	5	2-aminoantracene	2
TA102	cumene hydroperoxide	200	benzo[a]pyrene	10
TA1535	N-ethyl-N'-nitro-N-nitrosoguanidine	10	2-aminoantracene	2
TA1537	9-aminoacridine	50	2-aminoantracene	10
WP2 *uvrA*	N-ethyl-N'-nitro-N-nitrosoguanidine	5	2-aminoantracene	10

Incubation and sampling times: see Methods for Study T15570.

Results

Study validity: see Methods for Study T15570

Study outcome:

There was no precipitation of EMD 415722 on the agar plates. There was no clear toxicity to the bacteria. Each treatment with the positive controls led to a clear increase in revertant colonies, thus, showing the expected reversion properties of all strains and good metabolic activity of the S9 mix used. Negative and positive controls were within their expected ranges.

EMD 415722 showed no increase in the number of revertants of any bacterial strain with and without the addition of rat liver S9 mix (series 2 Table below, reproduced from the sponsor's submission). Thus, EMD 415722 was not mutagenic in this assay.

TABLE 3 / Series No.: 2

EMD 415722: Summary of the Mean Number of Revertant Colonies T15917

Test Material	Concentration [µg/plate]	+/- S9- Mix	Mean revertant colonies / plate TA 98	TA 100	TA 102
Solvent control		-	24	140	360
EMD 415722	50	-	24	144	371
	158	-	30	143	373
	500	-	25	140	397
	1580	-	35	142	388
	5000	-	39	152	426
Solvent control		+	34	196	368
EMD 415722	50	+	40	207	380
	158	+	44	224	378
	500	+	38	206	430
	1580	+	34	193	444
	5000	+	43	219	468
Positive controls	Name		DAUN	ENNG	CUM
	Conc.[µg/plate]	-	4	5	200
	Revert./plate		640	651	1354
	Name		2-AA	2-AA	B(a)p
	Conc.[µg/plate]	+	2	2	10
	Revert./plate		143	440	1171

Test Material	Concentration [µg/plate]	+/- S9- Mix	Mean revertant colonies / plate TA 1535	TA 1537	WP2 uvrA
Solvent control		-	22	9	48
EMD 415722	50	-	30	9	60
	158	-	24	8	54
	500	-	21	7	63
	1580	-	27	12	62
	5000	-	29	10	69
Solvent control		+	31	13	57
EMD 415722	50	+	33	17	67
	158	+	43	17	68
	500	+	30	17	72
	1580	+	37	13	72
	5000	+	41	17	71
Positive controls	Name		ENNG	9-AA	ENNG
	Conc.[µg/plate]	-	10	50	5
	Revert./plate		452	594	1288
	Name		2-AA	2-AA	2-AA
	Conc.[µg/plate]	+	2	10	10
	Revert./plate		136	188	139

DAUN Daunomycin
CUM Cumene hydroperoxide
B(a)p Benzo(a)pyrene
ENNG N-Ethyl-N'-nitro-N-nitroso-guanidine
2-AA 2-Aminoanthracene
9-AA 9-Aminoacridine

Study title: **EMD 415722 (Batch 2080) -Bacterial Mutagenicity Assay, *Salmonella Typhimurium* and *Escherichia Coli***

Key findings: EMD 415722 (hydroxocobalamin) at doses up to 5000 µg/plate was not mutagenic to strains of *S. typhimurium* and *E. coli* in the absence or presence of S9 mix.

NOTE: This study was completed with this batch of drug substance since this batch contains a higher level of impurities in the drug substance. According to the sponsor, the impurity levels in this batch are as follows:

Batch	2080
Manufacturing Date	July 30, 2004
Release Date (stated in study)	
used for Stability testing	**stored at 40°C/75% RH for 8 months**
Analysis Date	Aug 3, 2005 (Retest with the proposed HPLC method) (this is 12 months after manufactured)
[redacted]	[redacted]
Any other unspecified impurity	Max= [redacted] %
Total impurity content	

b(4)

Study no.: T16401
Vol. 12, Tab 4.2.3.7.6.4, T16401
Conducting laboratory and location: Institute of Toxicology Merck KGaA, 64271 Darmstadt, Germany
Date of study initiation: Oct 11, 2005
GLP compliance: yes
QA reports: yes
Drug, lot #, and % purity:
EMD 415722 (hydroxocobalamin), Batch 2080, Purity 89.8%
Vehicle: physiological saline

Methods

The methods were the same as described for Study T15570.

Strains/species/cell line:

Salmonella typhimurium TA98, TA100, TA102, TA1535, TA1537
Escherichia coli WP2 *uvrA*

Doses used in definitive study: 500, 889, 158, 1580, 2810 and 5000 µg/plate

Results

Basis of dose selection: see Methods for Study T15570.

Negative controls: physiological saline

Positive controls:

Strain	-S9		+S9	
	Compound	Dose (µg/plate)	Compound	Dose (µg/plate)
TA98	Daunomycin	4	2-aminoantracene	2
TA100	N-ethyl-N'-nitro-N-nitrosoguanidine	5	2-aminoantracene	2
TA102	cumene hydroperoxide	200	benzo[a]pyrene	10
TA1535	N-ethyl-N'-nitro-N-nitrosoguanidine	10	2-aminoantracene	2
TA1537	9-aminoacridine	50	2-aminoantracene	10
WP2 *uvrA*	N-ethyl-N'-nitro-N-nitrosoguanidine	5	2-aminoantracene	10

Incubation and sampling times: see Methods for Study T15570.

Results

Study validity: see Methods for Study T15570

Study outcome:

There was no precipitation of EMD 415722 on the agar plates. There was no clear toxicity to the bacteria. Each treatment with the positive controls led to a clear increase in revertant colonies, thus, showing the expected reversion properties of all strains and good metabolic activity of the S9 mix used. Negative and positive controls were within their expected ranges.

EMD 415722 showed no increase in the number of revertants of any bacterial strain with and without the addition of rat liver S9 mix (series 2 Table below, reproduced from the sponsor's submission). Thus, EMD 415722 was not mutagenic in this assay.

TABLE 3 / Series No.: 2

EMD 415722: Summary of the Mean Number of Revertant Colonies T16401

Test Material	Concentration [µg/plate]	+/- S9-Mix	Mean revertant colonies / plate TA 98	TA 100	TA 102
Solvent control		-	24	162	301
EMD 415722	500	-	28	141	329
	889	-	25	162	337
	1580	-	21	173	299
	2810	-	21	161	300
	5000	-	19	171	268
Solvent control		+	41	196	267
EMD 415722	500	+	48	206	261
	889	+	45	202	279
	1580	+	39	188	268
	2810	+	38	180	245
	5000	+	36	211	245
Positive controls	Name		DAUN	NaN3	CUM
	Conc.[µg/plate]	-	1	2	200
	Revert./plate		1133	695	1169
	Name		2-AA	2-AA	B(a)p
	Conc.[µg/plate]	+	2	2	10
	Revert./plate		119	358	630

Test Material	Concentration [µg/plate]	+/- S9-Mix	Mean revertant colonies / plate TA 1535	TA 1537	WP2 uvrA
Solvent control		-	16	8	44
EMD 415722	500	-	16	9	60
	889	-	13	10	49
	1580	-	15	11	50
	2810	-	16	7	54
	5000	-	24	14	54
Solvent control		+	22	24	59
EMD 415722	500	+	26	26	79
	889	+	25	23	79
	1580	+	19	29	88
	2810	+	25	26	76
	5000	+	24	16	81
Positive controls	Name		NaN3	9-AA	NQO
	Conc.[µg/plate]	-	2	50	2
	Revert./plate		580	410	1068
	Name		2-AA	2-AA	2-AA
	Conc.[µg/plate]	+	2	10	10
	Revert./plate		82	175	147

DAUN Daunomycin
CUM Cumene hydroperoxide
B(a)p Benzo(a)pyrene
NQO 4-Nitroquinoline-N-oxide
NaN3 Natriumazid
2-AA 2-Aminoanthracene
9-AA 9-Aminoacridine

Study title: **EMD 415722 (Cyanokit®) - In Vitro Mammalian Cell Gene Mutation Test (L5178Y TK$^{+/-}$)**

Key findings: Hydroxocobalamin was not mutagenic, at the TK locus (5-trifluorothymidine resistance) in mouse lymphoma cells using a fluctuation protocol at concentrations of 158 to 5000 µg/mL with or without S9.

Study no.: T15575
Vol. 11, Tab 4.2.3.3.1.3, T15575
Conducting laboratory and location: Institute of Toxicology, Merck KGaA, 64271 Darmstadt, Germany
Date of study initiation: April 22, 2003
GLP compliance: yes
QA reports: yes
Drug, lot #, and % purity:
EMD 415722 (hydroxocobalamin), Batch 2056, Purity 93.9%
Vehicle: physiological saline

Methods

Strains/species/cell line: L5178Y TK(+/-) mouse lymphoma cells
Each batch of frozen cells was purged of TK(-/-) mutants, checked for spontaneous mutant frequency and for absence of Mycoplasma.

Doses used in definitive study: 158, 500, 1580 and 5000 µg EMD 415722/mL medium

Basis of dose selection:

In a preceding range finding test, the relative survival was determined after exposure to various test material concentrations ranging between 5 and 5000 µg/mL. A reduction in the relative survival of the cells did not occur. Precipitation of EMD 415722 in the cell culture medium was not seen. A relevant change in the pH and the osmolarity of the culture medium was not detected.

At least four concentrations over an adequate concentration range should be employed. The highest concentration should precipitate in the culture medium or exhibit cytotoxicity. The cytotoxicity of the lowest concentration should usually correspond to that of the negative controls. Soluble test materials, if not toxic, should be tested up to a maximum concentration of 5000 µg/mL, 5 µL/mL, or 10 mM, respectively.

If cytotoxicity is the limiting factor for the selection of test material concentrations, usually five concentrations are established for the main study because toxicity may alter due to biological variability. Depending on the degree of toxicity in the main study, the highest or lowest concentration is omitted in the course of the mutagenicity experiment.

In addition to the biologic effects mentioned, the effects of the test material on the pH and the osmolarity of the cell culture medium were assessed

Negative controls: saline or DMSO

Positive controls:

	compound	final concentration
without S9	4-nitroquinoline N-oxide (NQO)	0.1 and 0.2 µg/mL
with S9	7,12-Dimethylbenz[a]anthracene (DMBA)	1.0, 2.0 or 3.0 µg/mL

Solvent for positive controls was dimethyl sulfoxide (DMSO)

Incubation and sampling times:

EMD 415722 was assayed for its ability to induce mutations at the TK locus (5-trifluorothymidine resistance) in mouse lymphoma cells using a fluctuation protocol. The study consisted of two independent experimental series, each conducted in the absence and presence of an exogenous metabolizing system (S9 mix from livers of rats pretreated with Aroclor 1254).

Each treatment, in the absence or presence of S9 mix, was performed in duplicate (single cultures only used for positive control treatments). On day 1 of the experiment, usually the following components (volumes in mL) were placed in each of a series of sterile disposable 50 mL centrifuge tubes (for the 3 hours incubations) or 75 cm^2 culture flasks (for the 24 hours incubations).

	Series – S9 mix 1st and 2nd Series	Series + S9 mix 1st Series	Series + S9 mix 2nd Series
10^7 cells in RPMI 5	19	18.5	19.5
Solvent, test article or positive control			
in aqueous solvent	0.2	0.2	0.2
in organic solvent	0.02	0.02	0.02
150 mM KCl	1	-	-
S9 mix	-	1.5	0.5

After incubation for 3 hours in the presence and 24 hours (1st series) or 3 hours (2nd series) in the absence of S9 mix at 37°C, the cells were washed with tissue culture medium and resuspended further in 10 mL RPMI 10 per tube. Cell densities were determined using a hemocytometer and the concentrations adjusted to 2 x 10^5/mL. Cells were transferred to flasks for growth through the expression period or were diluted to be plated for survival.

Plating for survival

Following adjustment of the cultures to 2 x 10^5 cells/mL after treatment, samples from these were diluted to 8 cells/mL, and 0.2 mL of that concentration were placed into each well of two 96-well microtiter plates (192 wells, at an average of 1.6 cells per well). The plates were incubated at 37°C in a humidified incubator gassed with 5% v/v CO2 in air until scorable (day 6 to day 10). Wells containing viable clones were, after staining with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenylbromide (MTT), identified by eye and counted.

Expression period
Cultures were maintained in flasks for 2 days (until day 3 of the experiment) during which the TK mutation would be expressed. Sub-culturing was performed as required with the aim of not exceeding 1 x 10^6 cells per mL and, where possible, retaining at least 1 x 10^7 cells/flask. From observations on recovery and growth of the cultures during the expression period, the cultures were selected to be plated for viability and TFT resistance (mutation assessment).

Plating for viability
At the end of the expression period (day 3), cell concentrations in the selected cultures were determined using a hemocytometer and adjusted to give 1 x 10^4/mL in readiness for plating for TFT resistance. Samples from these were diluted to 8 cells/mL, of which 0.2 mL of that concentration were placed into each well of two 96-well microtiter plates (192 wells at an average of 1.6 cells per well). The plates were incubated at 37°C in a humidified incubator gassed with 5% v/v CO_2 in air until scorable (day 7 to day 10). Wells containing viable clones were, after staining with MTT, identified by eye and counted.

Plating for TFT resistance
At the end of the expression period (day 3), the cell densities in the selected cultures were adjusted to 1 x 10^4/mL. TFT (300 µg/mL) was diluted 100-fold into these suspensions to give a final concentration of 3 µg/mL, of which 0.2 mL of each suspension was placed into each well of four 96-well microtiter plates (384 wells at 2 x 10^3 cells per well). Plates were incubated until scorable (day 10 to day 14) and wells containing clones were identified as indicated above and counted. At least for the negative and positive controls and, in case of a positive test material-induced effect, also for those cultures that showed the highest test material-induced effect, the mutation frequency is determined separately for small and large colonies in addition to the total mutation frequency.

Metabolic activator S9 mix was prepared using standard techniques after a single injection of Aroclor 1254 (500 mg/kg dissolved in Miglyol 812 oil) to male Wistar rats of 6-8 weeks of age. On day 5 to 7 after injection, they were sacrificed and livers removed and processed to create a S9 solution in phosphate buffered saline containing 20 mM HEPES.
Every S9 batch was tested for its metabolic activity by the use of specific substrates (2-aminoanthracene, benzo(a)pyrene, and 3-methylcholanthrene) requiring different enzymes of the P450-isoenzyme family. Clear increases in the number of revertants for various bacterial strains with all positive controls are used as an acceptance criterion for each S9 batch.

On the day of the experiment, glucose-6-phosphate (590 mM), NADP (30 mM), KCl (150 mM) and rat liver S9 were mixed at the ratio of 1:1:1:2. The mixture of S9 plus the added cofactors is termed S9 mix. For the treatment of the cells with test material, the final concentration of S9 in the cell culture medium was 3% in the lst and 1% in the 2nd experimental series, respectively. For all cultures treated in the presence of S9 mix, an aliquot of the mix was added to each cell culture to give a total of 20 mL. Cultures treated in the absence of S9 mix received an appropriate volume of 150 mM KCl.

Analysis

Determination of survival or viability

From the zero term of the Poisson distribution the probable number of clones/well (C) on microtiter plates in which there are empty wells (EW, without clones) and full wells (FW, with clones; TW = total wells = EW + FW) is given by:

$$C = -\ln [EW/TW]$$

The cloning efficiency (CE) in any given culture is:

$$CE = C/\text{Number of cells plated per well}$$

and, as an average of 1.6 cells per well were plated on all survival and viability plates,

$$CE = C/1.6$$

The percentage relative survival (%RS) in each test culture was determined by comparing cloning efficiencies in test and control cultures, thus:

$$\%RS = [CE\ (test)/CE\ (control)] \times 100$$

The relative total growth (RTG), an additional parameter taking the cytotoxicity of the test material at the different stages of this mutagenicity assay into consideration, is calculated as (cf. Tables section):

[Number of cells after treatment] x [Number of cells before selection] x [CE of cells at selection]

Determination of mutant frequency

It is common to express mutant frequency (MF) as "mutants per 10^6 viable cells". In order to calculate this, the cloning efficiencies of both mutant and viable cells in the same culture were calculated,

$$MF = [CE\ (mutant)/CE\ (viable)] \times 10^6$$

From the formulae given in 3.3.1 and with the knowledge that 2×10^3 cells were plated/well for mutation to 5-trifluorothymidine resistance,

$$CE\ (mutant) = C\ (mutant)/2 \times 10^3$$
$$CE\ (viable) = C\ (viable)/1.6$$

where, in each case, $C = -\ln [EW/TW]$

Therefore, $MF = [C\ (mutant) / 2 \times 10^3] / [C\ (viable) / 1.6] \times 10^6$

$= [C\ (mutant) / C\ (viable)] \times [1.6 \times 10^6 / 2 \times 10^3]$

$= [-\ln [EW/TW]\ (mutant) / -\ln [EW/TW]\ (viable)] \times 800$

Results

Study validity (comment on replicates, counting method, criteria for positive results, etc.):

The assay was considered valid if the following criteria were met:

- The mean mutant frequencies in the negative (solvent) control cultures fell within the normal range (not more than two times the historical mean value)
- at least one concentration of each of the positive control chemicals is assessed as positive or mutagenic in this test system.

Evaluation criteria

The effects of the test material upon the mutation frequency are defined as

- "No effect" or "no increase" in the mutation frequency if the mean frequency of the parallel incubations of a given test material concentration is less than 2.0-fold above the mean of the actual negative controls or the mean mutation falls within the historical range of the negative controls.
- "Clear effect" or "clear increase" in the mutation frequency if the test material induces at least a 3.0-fold increase above the mean of the actual negative controls and the mean mutation frequency for a given test material concentration is at least 1.5-fold above the highest value of the historical negative controls.
- All other results are defined as a "weak effect" or a "weak increase" of the mutation frequency.

Test materials are assessed as negative or non-mutagenic in this test system if

- the assay is considered valid and
- no effect (no increase in the mutation frequency) occurs in the two experimental series performed or
- a weak effect (weak increase) occurs in one series and no effect (no increase) in the other series of experiments.

Test materials are assessed as positive or mutagenic in this test system if

- the assay is considered valid and
- a clear effect (clear increase in the mutation frequency) occurs at similar concentrations of the test material in the two experimental series performed, or
- a clear effect (clear increase) occurs in one series and a weak effect (weak increase) in the other series of experiments at identical concentrations, or
- weak effects (weak increases) occur dose-dependently (over at least two test material concentrations) and reproducibly at identical concentrations in the two experimental series performed.

Study outcome:

Solubility and toxicity

In a preceding range finding test, the relative survival was determined after exposure to various test material concentrations ranging between 5 and 5000 µg/mL. A reduction in the relative survival of the cells did not occur. Precipitation of EMD 415722 in the cell culture medium was not seen. A relevant change in the pH and the osmolarity of the

culture medium was not detected. Clear cytotoxic effects of EMD 415722 on the lymphoma cells were observed only at the highest concentration in the first series of experiments in the absence of S9 mix.

Mutation
A summary of the results (mean values) is shown in Table 1.
Negative (solvent) and positive control treatments were included in each mutation experiment in the absence and presence of S9 mix. Mutant frequencies in negative control cultures fell within normal ranges, and clear increases in mutation were induced by the positive control chemicals 4-nitroquinoline N-oxide (without S9 mix) and 7,12-dimethylbenz(a)anthracene (with S9 mix). Therefore, the study was accepted as valid.

Four EMD 415 722 concentrations ranging from 158 to 5000 µg/mL were tested in the absence or presence of S9 mix. No precipitation and only weak cytotoxic effects of the test material at the highest test material concentration in the absence of S9 mix were observed. The doses tested were selected to determine viability and mutagenicity (5-trifluorothymidine (TFT) resistance) 2 days after treatment.

In the absence of S9 mix, the individual mutation frequency of the solvent controls ranged from 87.2 x 10^{-6} to 197 x 10^{-6} in both experimental series performed. The mutation frequencies of cells treated with the positive control, NQO, increased to values ranging from 279 x 10^{-6} to 781 x 10^{-6}. The cultures treated with the different concentrations of EMD 415722 showed mutation frequencies between 44.4 x 10^{-6} and 262 x 10^{-6}. The mean mutation frequencies (presented in Table 1) for the EMD 415722-treatment groups and the solvent controls were not "relevantly different." In the presence of S9 mix, the individual mutation frequency of the solvent controls ranged from 54.6 x 10^{-6} to 95.7 x 10^{-6} in both experimental series. The mutation frequencies of cells treated with the positive control DMBA were increased to values ranging from 238 x 10^{-6} to 1099 X 10^{-6}. The cultures treated with the different concentrations of EMD 415722 had mutation frequencies between 41.9 x 10^{-6} and 151 x 10^{-6} similar to the solvent control. In conclusion, there was no relevant increase in the mutation frequency induced by EMD 415722. Thus, under these assay conditions, EMD 415722 was not mutagenic.

6. Table 1: Summary of results (of the data from Tables 2 and 3)

Without S9 mix (EZ1473/1486)

1st Experimental series					2nd Experimental series				
Test material	[µg/mL]	RS[a] [%]	RTG[b] [%]	MF[c]	Test material	[µg/mL]	RS[a] [%]	RTG[b] [%]	MF[c]
Solvent	0	100	100	179	Solvent	0	100	100	97.8
EMD 415 722					EMD 415 722				
	158	122	105	151		158	93.9	108	56.0
	500	121	104	134		500	101	99.7	81.9
	1580	123	67.8	171		1580	107	98.1	62.2
	5000	110	34.4	256		5000	51.7	66.2	128
NQO	0.10	108	97.1	447	NQO	0.10	80.2	81.9	279
	0.20	100	73.6	781		0.20	47.0	55.1	350

With S9 mix (EZ 1474/1487)

1st Experimental series					2nd Experimental series				
Test material	[µg/mL]	RS[a] [%]	RTG[b] [%]	MF[c]	Test material	[µg/mL]	RS[a] [%]	RTG[b] [%]	MF[c]
Solvent	0	100	100	87.9	Solvent	0	100	100	55.6
EMD 415 722					EMD 415 722				
	158	98.6	81.5	117		158	91.9	111	49.1
	500	116	75.3	114		500	99.0	90.9	56.4
	1580	92.3	69.5	142		1580	99.0	101	57.5
	5000	92.4	74.5	113		5000	106	107	42.8
DMBA	2.00	54.8	50.4	824	DMBA	1.00	77.5	73.1	238
	3.00	3.70	4.46	848		2.00	3.10	4.67	1099

a: Relative Survival (CE of cells after treatment)
b: Relative Total Growth
c: 5-TFT Mutant Frequency per 10^6 viable cells
EZ: Internal study number
NQO: 4-Nitroquinoline N-oxide
DMBA 7,12-dimethylbenz[a]anthracene

Without S9 mix (2nd experimental series; EZ 1486)

Test material [µg/mL]		Number of cells after treatment absolute[a]	mean%	Number of cells before selection absolute[a]	mean%	CE of cells after treatment (RS) absolute[b]	mean%	CE of cells at selection absolute[b]	mean%	Relative Total Growth (RTG) absolute[c]	mean%
Solvent											
0	A	9.13	100	65.5	100	1.25	100	1.21	100	721	100
	B	9.18		62.8		1.28		1.28		737	
EMD 415 722											
158	A	9.00	97.4	73.5	117	1.19	93.9	1.17	95.5	772	108
	B	8.83		76.0		1.19		1.21		809	
500	A	9.78	101	61.3	103	1.20	101	1.21	96.2	722	99.7
	B	8.65		71.3		1.36		1.19		731	
1580	A	9.53	96.5	76.0	115	1.16	107	1.08	86.5	784	96.1
	B	8.13		71.3		1.54		1.06		617	
5000	A	9.45	102	49.3	78.0	0.717	51.7	1.05	83.1	489	66.2
	B	9.25		50.8		0.588		1.01		476	
NQO 0.10		9.05	98.9	69.0	108	1.01	80.2	0.967	77.8	604	81.9
0.20		8.95	97.8	50.3	78.4	0.594	47.0	0.903	72.7	406	55.1

a: x 10^5 / ml
c: {Number of cells after treatment} x {Number of cells before selection} x {CE of cells at selection}
b: - ln {empty wells / total wells}
A, B ... = Replicate cultures
CE: Cloning efficiency
EZ: Internal study number
NQO: 4-Nitroquinoline N-oxide

Table 2 continued on next page

With S9 mix (2nd experimental series; EZ 1487)

Test material [µg/mL]		Number of cells after treatment absolute[a]	mean%	Number of cells before selection absolute[a]	mean%	CE of cells after treatment (RS) absolute[b]	mean%	CE of cells at selection absolute[b]	mean%	Relative Total Growth (RTG) absolute[c]	mean%
Solvent											
0	A	8.60	100	56.5	100	1.23	100	1.33	100	647	100
	B	9.60		55.0		0.951		1.18		624	
EMD 415 722											
158	A	9.25	103	57.0	99.6	0.923	91.9	1.27	108	668	111
	B	9.50		54.0		1.08		1.45		746	
500	A	9.20	102	44.8	82.2	1.11	99.0	1.38	108	570	90.9
	B	9.43		46.8		1.05		1.33		586	
1580	A	10.2	106	48.8	86.2	1.03	99.0	1.38	110	689	101
	B	9.08		47.3		1.13		1.39		597	
5000	A	10.5	103	47.8	88.4	1.10	106	1.50	117	751	107
	B	8.18		50.8		1.21		1.45		604	
DMBA 1.00		9.68	106	37.5	67.3	0.843	77.5	1.26	100	456	73.1
2.00		7.65	84.1	17.2	30.9	0.0338	3.10	0.222	17.6	29.2	4.67

a: x 10^5 / ml
b: - ln {empty wells / total wells}
c: {Number of cells after treatment} x {Number of cells before selection} x {CE of cells at selection}
A, B ... = Replicate cultures
EZ: Internal study number
CE : Cloning efficiency
DMBA 7,12-dimethylbenz[a]anthracene

Without S9 mix (2nd experimental series; EZ 1486)

Test material [µg/mL]		MF[a] total individual	MF[a] total mean	MF[a] small individual	MF[a] small mean	MF[a] large individual	MF[a] large mean	MF small / MF large
Solvent								
0	A		97.8		49.7		49.9	1.00
	B							
EMD 415 722								
158	A		56.0					
	B							
500	A		81.9					
	B							
1580	A		62.2					
	B							
5000	A		128					
	B							
NQO	0.10		279					3.21
	0.20		350					3.27

b(4)

a: 5-TFT Mutant Frequency per 10^6 viable cells
A, B ...: Replicate cultures
EZ: Internal study number
NQO: 4-Nitroquinoline N-oxide

Table 3 continued on next page

With S9 mix (2nd experimental series; EZ 1487)

Test material [µg/mL]	MF[a] total individual	MF[a] total mean	MF[a] small individual	MF[a] small mean	MF[a] large individual	MF[a] large mean	MF small / MF large
Solvent							
0		55.6	/	7.46	/	47.2	0.159
EMD 415 722							
158		49.1					
500 A		56.4					
B							
1580 A		57.5					
B							
5000 A		42.8					
B							
DMBA 1.00		238	/		/		1.14
2.00		1099					3.25

b(4)

a: 5-TFT Mutant Frequency per 10^6 viable cells
A, B ...: Replicate cultures
EZ: Internal study number
DMBA 7,12-dimethylbenz[a]anthracene

End of Table 3

Range finder

Without S9-Mix (EZ1464), Treatment 3 h.

Test Material	Conc. [µg/mL]	Relative Survival [%] [a]
Solvent (DMSO)		100
EMD 415 722	5.00	103
	15.8	97.9
	50.0	105
	158	105
	500	103
	1580	102
	5000	89.9

Without S9-Mix (EZ1464), Treatment 24 h.

Test Material	Conc. [µg/mL]	Relative Survival [%] [a]
Solvent (DMSO)		100
EMD 415 722	5.00	114
	15.8	89.3
	50.0	83.3
	158	72.0
	500	104
	1580	84.6
	5000	85.8

With S9-Mix (EZ1464), Treatment 3 h.

Test Material	Conc. [µg/mL]	Relative Survival [%] [a]
Solvent (DMSO)		100
EMD 415 722	5.00	95.2
	15.8	94.6
	50.0	92.6
	158	106
	500	96.5
	1580	90.8
	5000	110

a: Day 2 of Experiment (Survivor 1)
EZ: Internal study number

Study title: EMD 415722 (Cyanokit®) -Micronucleus Test in Rats after Intravenous Administration

Key findings: EMD 415722 (hydroxocobalamin) administered to male rats at doses up to 140 mg/kg, sufficient to induce clinical signs of toxicity, did not induce micronuclei in rat bone marrow polychromatic erythrocytes and did not alter the ratio of normochromic to polychromic erythrocytes. Therefore, hydroxocobalamin was not clastogenic in this assay.

Study no.: T15574
Vol. 11, Tab 4.2.3.3.2.1 T15574
Conducting laboratory and location: Institute of Technology, Merck KGaA, 64271 Darmstadt, Germany
Date of study initiation: April 3, 2003 (end of experimental phase was May 5, 2003)
GLP compliance: yes, (signed Oct 10, 2005, ~2.5 yrs after experimental phase ended)
QA reports: yes (signed Oct 10, 2005, ~2.5 yrs after study experimental phase ended)
Drug, lot #, and % purity:
EMD 415722 (hydroxocobalamin), Batch 2056, Purity 93.9%
Vehicle: physiological saline

Methods

Strains/species/cell line: male Wistar rats (———WU), 6 weeks of age, n=5/dose b(4)

Doses used in definitive study: 0, 14, 44.3 and 140 mg/kg body weight, administered IV (since it would be given to humans in this manner)
Doses were administered once at a volume of 10 mL/kg. The rats of the negative control group were treated intravenously with 10 mL/kg body weight physiological saline. Animals of the positive control group received an oral dose of 16.5 mg cyclophosphamide/kg body weight.

Basis of dose selection:

The highest EMD 415722 dose given in the present study was selected to produce signs of toxicity but no mortality. In a preliminary dose-finding experiment involving a total of 8 males, the administration of 140 mg EMD 415722/kg body weight led to signs of toxicity, such as forced breathing, incomplete eyelid closure, abdominal position, staggering in one animal and body weight loss. Mortality was not observed, up to and including the third day after treatment. For these reasons, the dose of 140 mg EMD 415722/kg body weight was selected as the highest doses for rats in the main study of this investigation. The mid and low dose was obtained by half-log dilutions.

Negative controls: physiological saline

Positive controls: cyclophosphamide (———®), 16.5 mg/kg administered orally

b(4)

Procedure
EMD 415 722 was given once intravenously to male rats. Doses were administered once at a volume of 10 mL/kg. The rats of the negative control group were treated intravenously with 10 mL/kg body weight physiological saline. Animals of the positive control group received an oral dose of 16.5 mg cyclophosphamide/kg body weight. Bone marrow smears were prepared from one femur of each animal at 24 or 48 hours after dose administration and stained with Giemsa's solution. For the 140 mg/kg dose, bone marrow smears were prepared at both 24 and 48 hours after treatment. For the lower dose groups, as well as for the positive and negative control groups, bone marrow smears were prepared at 24 hour after treatment.

Dose Groups

Group	Test material	Dose[a] [mg/kg]	Preparation time[h][b]	Males[c]
A	Solvent	0.0	24	5
B	EMD 415 722	14.0	24	5
C	EMD 415 722	44.3	24	5
D	EMD 415 722	140	24	5
E	EMD 415 722	140	48	5
F	CPA	16.5	24	5

Analysis
One slide from each animal preparation was coded. The number of polychromatic erythrocytes with micronuclei per 2000 polychromatic erythrocytes per animal was determined. The quotient of normochromatic to polychromatic erythrocytes was calculated based on the analysis of 1000 erythrocytes per animal. The micronucleated normochromatic erythrocytes were registered when scoring the polychromatic erythrocytes. The number of micronucleated normochromatic erythrocytes per 1000 erythrocytes was then calculated with the aid of the normochromatic to polychromatic erythrocytes quotient

For all groups, mean values were calculated for the following parameters:

NCE/PCE number of normochromatic erythrocytes (NCE) / number of polychromatic erythrocytes (PCE) / animal

MN-NCE number of micronuclei-containing cells / 1000 NCE / animal

MN-PCE number of micronuclei-contaning cells /1000 PCE /animal

Results

Study validity:

Round particles with about 1/20 to 1/5 the diameter of an erythrocyte that stained violet, like nucleic material, were scored as micronuclei. They were differentiated from granules

by thorough examination at different optical levels. Only erythrocytes with a distinct bluish touch were evaluated as polychromatic.

The results were classified as mutagenic or non-mutagenic according to the following rules:

> A positive effect is defined by the occurrence of mean MN-PCE values of a treatment group that are statistically significantly higher than those of the actual negative control. A prerequisite for this was that the values also be greater than historical negative controls.
>
> Significant positive effects must occur in the actual positive control group.
>
> If there is no positive effect of the test material in the main study, then the substance is defined as a non-mutagen in this test system. The study is terminated.
>
> If a positive effect occurs in a single test group (i.e. dose-independently), a repeat experiment has to be considered. In case that no positive effects occur in that experiment the test material is defined as a non-mutagen. The single positive effect of the first experiment is interpreted as a randomly occuring event of no biological significance.
>
> A test material is defined as mutagenic in this system if dose-related and/or single, reproducible (in independent experiments) positive effects occur. Establishment of dose-dependent effects of the test material is preferable. For this reason, if a positive effect occurs in a study in which a single, limit dose of 2000 mg/g body weight was used, 3 different doses need to be administered in the supplemental experiment. The above mentioned criteria for a negative or positive test result also apply for this experimental design. If borderline cases occur, the decision on further procedures should be based on a scientific evaluation of all available results including the toxicokinetic data.

Study outcome:

Clinical Findings:
The highest test material dose induced some clinical signs of toxicity and, in addition, a weak decrease in body weight.

Clinical findings

Symptoms	Dose [mg/kg body weight]			
	0	14.0	44.3	140
Dyspnea	0/5	0/5	1/5	8/10
Ptosis	0/5	0/5	1/5	8/10
Abdominal position	0/5	0/5	0/5	7/10
Lateral position	0/5	0/5	0/5	2/10
Staggering	0/5	0/5	0/5	1/10
Reddish urine	0/5	5/5	5/5	10/10

Micronucleated Cells:
The saline control values were all in the expected range predetermined as historical controls of the laboratory. For the positive control, cyclophosphamide, there was statistically significant increase in polychromatic erythrocytes with micronuclei ($p<0.01$).

No statistically significant or biologically relevant increase in the number of polychromatic erythrocytes with micronuclei (MN-PCE) was observed for any dose of hydroxocobalamin. The ratio of normochromatic to polychromatic erythrocytes was not affected by hydroxocobalamin. Thus hydroxocobalamin was not mutagenic in this assay.

study no.: T15574_1 MICRONUCLEI-CONTAINING CELLS: MEAN VALUES Table - 1 -
species: rat
application: iv/oral

group	test material	dose [mg/kg]	prep. time [h]	n	NCE/PCE mean	NCE/PCE SD	NCE/PCE p	NCE/PCE sig	MN-NCE mean	MN-NCE SD	MN-NCE p	MN-NCE sig	MN-PCE mean	MN-PCE SD	MN-PCE p	MN-PCE sig
A	SOLVENT	0.0	24	5	0.94	0.12			0.22	0.31			1.90	1.14		
B	EMD 415 722	14.0	24	5	1.04	0.23	0.899	n.s.	0.00	0.00	1.000	n.s.	2.10	0.82	0.381	n.s.
C	EMD 415 722	44.3	24	5	1.15	0.29	0.378	n.s.	0.16	0.23	0.738	n.s.	1.60	0.82	0.695	n.s.
D	EMD 415 722	140.0	24	5	0.74	0.07			0.12	0.27	0.361	n.s.	2.10	1.85	0.547	n.s.
E	EMD 415 722	140.0	48	5	0.95	0.15	1.000	n.s.	0.20	0.27	0.658	n.s.	2.60	1.88	0.361	n.s.
F	CPA	16.5	24	5	1.32	0.28	0.032	*	0.43	0.45	0.262	n.s.	31.10	8.83	0.004	**

Historical Controls

Study number	Mean micronuclei containing cells per 1000 polychromatic erythrocytes [a]			
	Solvent controls		Positive controls [b]	
	males	females	males	females
Historical Controls:				
Mean ± SD:	1.40 ± 0.75	1.20 ± 0.77	17.5 ± 6.55	10.6 ± 5.43
Range:	0.40 - 3.80		6.60 - 35.4	4.40 - 23.0

a: Mean number of micronuclei-containing cells per 1000 polychromatic erythrocytes from the last 25 studies performed in the laboratory.
b: CPA (16.5 mg/kg)
nd: Not determined

Reviewer's Comment: The response of MN-PCE in Group E at 48 hours after treatment with 140 mg/kg is a borderline case. The variation is large enough to be considered

nonsignificant (one rat of 5, had a value of 5.5, and there was a rat in the 24 hr 140 mg/kg group with a value of 5.0), but the mean lies just above the historical solvent upper limit. This dose is near the high dose of hydroxocobalamin used in the dog efficacy study (150 mg/kg) and therefore would be close to the proposed human dose. In the Toxicological studies, bone marrow was a target organ, but there were no obvious alterations in cellularity or ratio of erythroid:myeloid cells.

2.6.6.5 Carcinogenicity

Carcinogenicity studies were neither conducted nor required for this indication. This product is expected to be used acutely, once, as a lifesaving treatment for cyanide poisoning.

2.6.6.6 Reproductive and developmental toxicology

Reviewer's Comments: Embryo-fetal toxicity studies were conducted with rats and rabbits in 1974. These studies do not meet current GLP and ICH guidelines and therefore cannot be used to support the reproductive safety of the proposed product. In addition there were no studies to address potential toxic effects on male fertility and postnatal development. The numerous deficiencies in the two embryo-fetal studies include the following:

- Characterization of hydroxocobalamin and control solvent
- Description of methodology and analysis
- Lack of appropriate animal numbers
- Lack of adequate doses (no indication of maternal toxicity)
- Quantification and characterizing reproductive and developmental parameters (some data not expressed per litter, group together as per treatment)
- Lack of original data, only summary tables

Since the standard battery of reproduction and developmental toxicology studies are deficient or have not been completed, the reviewer suggests that these studies be completed as part of a Phase 4 commitment.

Fertility and early embryonic development

Study title: **Embryofetal and Perinatal Toxicity Study in the Rat (** [redacted]

Key study findings: Although the study results suggest that hydroxocobalamin was not embryotoxic or teratogenic in rats at doses of 5 or 50 mg/kg, the study is not adequate by current standards.

Study no.: III.C.1:
Vol. 12, Tab 4.2.3.7.7.6.1 III.C.1

Conducting laboratory and location: [redacted]

Date of study initiation: during 1974, before GLP practices established
GLP compliance: no
QA reports: no
Drug, lot #, and % purity:
Hydroxocobalamin, batch and purity were not reported
Vehicle: solvent is not identified

b(4)

Methods

Male and female Wistar rats (300 ± 25 g) were paired overnight for mating. Females with positive spermatozoa in a vaginal smear (gestation day 1). From day 1 to day 19 (for 6 of 7 days of the week), females were administered subcutaneous injections of isotonic saline, solvent, 5 mg/kg (2.5 mg/mL) or 50 mg/kg (30 mg/mL) hydroxocobalamin. On day 19, animals were sacrificed, and reproductive tissues, appendices and fetuses were examined, removed and weighed. The uterus was stained by Salewski's method to detect implantations.

Results

There were no descriptions of clinical signs, maternal weight or food intake.

Fertility parameters (mating/fertility index, corpora lutea, preimplantation loss, etc.):

Reproductive Parameters in Rats Treated with Hydroxocobalamin

	Saline	Solvent	Hydroxocobalamin	
			5 mg/kg	50 mg/kg
Number of mated females	15	18	25	17
Number of pregnant females (%)	15 (100%)	15 (83.3%)	15 (60%)	15 (88.2%)
There was no explanation for the low (60%) pregnancy rate in the 5 mg/kg hydroxocobalamin group.				
Number of Implantations	177	182	172	184
Number of resorptions (%)	14 (7.9%)	22 (12.1%)	15 (8.7%)	30 (16.3%)
Mean number of fetuses per dam at full term	10.86 ± 0.84	10.73 ± 0.75	10.46 ± 0.89	11.00 ± 0.94
The number of abortions, if occurred were not providedin the study report. With the exception of fetuses per dam at term, the number of implantation and resorptions/dam were not provided. On face value neither dose of hydroxocobalamin had embryotoxic effects.				
Mean fetus weight (g)	2.45 ± 0.14	1.37 ± 0.12*	2.03 ± 0.20	2.16 ± 0.22
Mean placenta weight (g)	410 ± 13	345 ± 22*	398 ± 15	390 ± 17
Mean fetus/placenta weight ratio	6.01 ± 0.37	3.93 ± 0.25*	4.96 ± 0.39	5.47 ± 0.51
Reduced fetal, placenta, and fetal/placenta ratio were noted in the solvent treated group. There was no explanation for this effect. The Sponsor noted that the hydroxocobalamin group received a				

similar volume of solvent.				
Mean weight of appendix/fetus at full term (g)	1.81 ± 0.10	1.45 ± 0.13#	1.75 ± 0.11	1.61 ± 0.14
The appendix weight was also lower in the solvent group				

* p≤1% versus the absolute control animals
# p≤2% versus the absolute control animals

There were no fetal malformations observed upon gross examination or after alizarine staining of the skeletons, or after histological analysis (but tissues processed for analysis were not identified).

Reviewer's Comment: These studies conducted in 1974 do not meet our current GLP and Reproductive toxicology standards as presented in ICH-S5A, -S5B, and -S5B(M) Guidances for Industry. The reproductive toxicology studies should be repeated according to these guidances at appropriate doses to ensure maternal toxicity at the highest dose. Toxicokinetic parameters should be obtained to provide exposure comparisons.

Study title: **Embryofetal and Perinatal Toxicity Study in the Rabbit**

Key study findings: Although the study results suggest that hydroxocobalamin was not embryotoxic or teratogenic in rabbits at a dose of 50 mg/kg, the study is not adequate by current standards.

Study no.: III.C.2:
Vol. 12, Tab 4.2.3.7.7.6.2 III.C.2
Conducting laboratory and location:

b(4)

Date of study initiation: during 1974, before GLP practices established
GLP compliance: no
QA reports: no
Drug, lot #, and % purity:
Hydroxocobalamin, batch and purity not reported
Vehicle: solvent is not identified

Methods

Male and female rabbits ("conventional, common-strain;" 2.0 to 2.5 kg) were monitored for mating. From gestation day 5 to day 28 (for 6 of 7 days of the week), females were administered subcutaneous injections of isotonic saline, solvent, or 50 mg/kg (30 mg/mL) hydroxocobalamin. On day 28, animals were sacrificed, and reproductive tissues and fetuses were examined, removed and weighed. The uterus was stained by Salewski's method to detect implantations.

Results

There were no descriptions of clinical signs, maternal weight or food intake.

Fertility parameters (mating/fertility index, corpora lutea, preimplantation loss, etc.): In non-pregnant females, no implantations were observed. There was no effect of hydroxocobalamin on implantation or embryotoxicity. No malformations were observed by direct macroscopic examination or after skeletal staining with alizarine sulfonate. No teratogenicity was observed. There was an increase in fetal and placenta weight in the hydroxocobalamin group, but no change in the fetal placenta ratio. The Sponsor attributes the increase weight to a stimulant effect of hydroxocobalamin on protein anabolism.

Reproductive Parameters in Rabbits Treated with Hydroxocobalamin

	Saline	**Solvent**	**Hydroxocobalamin 50 mg/kg**
Number of mated females	5	5	11
Number of pregnant females (%)	4 (80%)	4 (80%)	9 (82%)
Number of Implantations	27	29	55
Number of resorptions (%)	4 (14.8%)	2 (6.9%)	5 (9.1%)
Mean number of fetuses per dam at full term	6.75 ± 0.7	7.25 ± 0.9	6.11 ± 0.7
Mean fetus weight (g)	32.3 ± 1.8	34.6 ± 0.9	36.5 ± 0.7*
Mean placenta weight (g)	5.79± 0.31	5.69 ± 0.14	6.17 ± 0.17*
Mean fetus/placenta weight ratio	5.62 ± 0.17	6.11 ± 0.20	6.08 ± 0.16

* $p \leq 1\%$ versus the absolute control animals
# $p \leq 2\%$ versus the absolute control animals

Reviewer's Comment: These studies conducted in 1974 do not meet our current GLP and Reproductive toxicology standards as presented in ICH-S5A, -S5B, and -S5B(M) Guidances for Industry. The reproductive toxicology studies should be repeated according to these guidances at appropriate doses to ensure maternal toxicity at the highest dose. Toxicokinetic data should also be obtained.

2.6.6.7. Local Tolerance

There were no separate studies on local tolerance. The Sponsor did not indicate abnormal clinical or histopathological findings for injection sites in the dog toxicology studies. In the rat studies following intraperitoneal injection, some abdominal pathological findings were attributed to the injection.

Hypersensitivity reactions occurred in the dog studies, evidenced by swelling of regions of the head and ears and the presence of wrinkles or wheals soon after intravenous administration of hydroxocobalamin. These reactions resolved within a few hours or by the following day, somewhat dependent on dose administered. They were not apparent with dosing on subsequent days. In humans, cases of hypersensitivity reactions to the administration of vitamin B12 derivatives (hydroxocobalamin and cyanocobalamin) have been documented (see Clinical Review).

2.6.6.8 Special toxicology studies

Study title: **EMD 415722(Cyanokit®2.5g) -Evaluation of In Vitro Phototoxicity on Balb-c 3T3 Fibroblasts Using the Neutral Red Uptake Assay**

Key study findings: Hydroxocobalamin was not phototoxic to *in vitro* cultures of mouse fibroblasts at doses up to 1000 µg/mL with or without exposure to UVA light wavelengths.

Study no.: 70/212
Vol. 12, Tab 4.2.3.7.7.2.1
Conducting laboratory and location: [redacted] b(4)

Date of study initiation: May 4, 2005
GLP compliance: yes (signed Oct 7, 2005)
QA report: yes (signed July 10, 2005
Drug, lot #, and % purity:
EMD 415722 (hydroxocobalamin), Batch 2066, Purity 96.2%
Vehicle: physiological saline diluted in phosphate buffered saline

Methods

Balb/c3T3 mouse fibroblast cells, seeded into 96 well microtiter plates, were treated with EMD 415722 or the positive control chemical (chlorpromazine, CPZ) in the presence and absence of Ultraviolet A light (UV-A). Doses (6 wells/dose) of EMD 415722 were:
EMD 415722:
+UV-A: 0.3160, 1.000, 3.160, 10.00, 31.60, 100.0, 316.0, 1000 µg/mL
-UV-A: 0.3160, 1.000, 3.160, 10.00, 31.60, 100.0, 316.0 1000 µg/mL
CPZ, +UV-A: 0.1, 1, 10, 100 µg/mL

CPZ: µg
+UV-A: 0.1, 1, 10, 100 µg/mL
-UV-A: 1, 10, 100, 1000 µg/mL

Solvent control (physiological saline in PBS) treatments and blanks were also included on each plate. The cultures were treated for 1 hour at 3°C prior to irradiation. One set of plates were exposed to 5 J/cm2 UVA (315-400 nm, 108+ minutes, filtered out UVB emission) and a second set of plates were kept in the dark for same duration. After irradiation, the media was aspirated from each well, cells washed with a suitable volume of PBS, and finally 0.2 mL medium was added to each well. The plates were then incubated for 20 ± 2 hours at 37°C in a humidified atmosphere of 5% CO2 in air. At the end of incubation period, cytotoxicity was assessed by the Neutral Red Uptake assay. A photo-irritation factor (PIF) was calculated as follows:

$$\text{PIF} = \frac{IC_{50} \text{ in the absence of UV-A}}{IC_{50} \text{ in the presence of UV-A}}$$

Results

Chlorpromazine induced an acceptable positive response with a PIF value of 27.2. In the negative controls, there was a low variability in OD values between the treatment replicates (coefficient of variance < 20%). The assay was therefore considered valid.

Treatment of cultures with hydroxocobalamin did not alter cell survival, either in the absence or the presence of UVA light. Survival at the maximum concentration of 1000 µg/mL was 96% in the absence of UVA light and 102% in the presence of UVA light, respectively. The survival curves were similar and there were no significant differences in Neutral Red Uptake in the presence of UVA when compared to those in the absence of UV light. The cell survival at the highest concentration tested (1000 µg/mL) was more than 50% and hence IC_{50} and PIF values could not be calculated. The IC_{50} and PIF calculations are given below. According to the OECD guidelines and under the conditions employed in this study, hydroxocobalamin was not phototoxic in this *in vitro* test system.

Test article	IC_{50} absence of UV-A (µg/mL)	IC_{50} presence of UV-A (µg/mL)	PIF Value
EMD 415722 (CYANOKIT® 2,5g)	*	*	*
Chlorpromazine	30.036	1.150	27.169**

* The cell survival at the highest concentration tested (1000 µg/mL) was more than 50% and hence IC_{50} and PIF values could not be calculated.
** PIF ≥ 5, therefore positive control response was acceptable.

Reviewer's Comments: This assay determines cell viability. Neutral red is a weak cationic supravital dye that penetrates cell membranes by non-ionic passive diffusion. Neutral red dye accumulates in the lysosomes, with a greater amount of dye uptake as an indication of cytotoxicity. Toxicity measures of this assay reflect only the most serious degree, that of cell death. Phototoxicity may also cause less severe cellular reactions in skin resulting in erythema, swelling, and eschar formation, altered DNA (thymidine dimmer, adduct formation) which may lead to mutagenesis and tumors, not detectable by this assay. This assay did not test UVB wavelengths, nor does it consider other types of cells in the skin.

2.6.6.9 Discussion and Conclusions

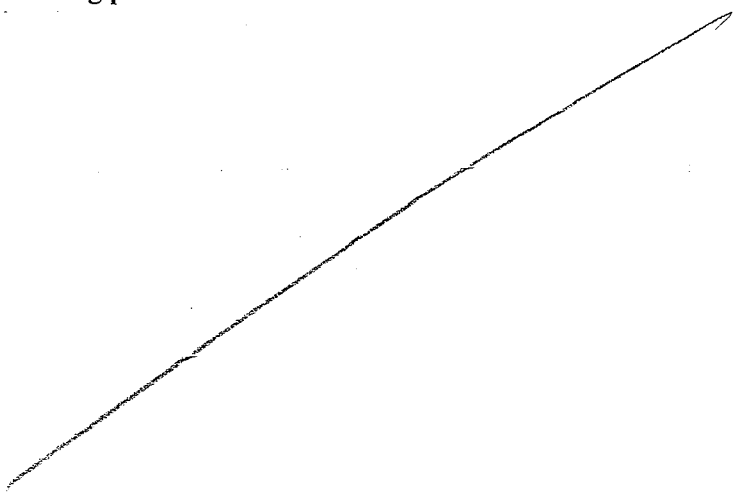
General Toxicology: Single and repeated dose toxicological studies were performed in rats and dogs. Many of these studies did not satisfy our recommended guidelines in terms of number of animals, gender of animals, length of treatment, and parameters measured. However, in total, the major toxicities could be identified.

In the single dose rat studies, hydroxocobalamin was administered intraperitoneal at doses of 75 to 1000 mg/kg. The major clinical signs in rats included dyspnea, locomotor disturbance, piloerection, incomplete eyelid closure, sunken flank and reddish urine and reddish skin. The major organ toxicities were accumulation of fluid in the lungs at 1000 mg/kg (highest dose) and death in some animals (1000 mg/kg), discolored and congested liver, discolored organs and tissues at ≥300 mg/kg including skin, abdominal fat, testes, and epididymides.

In the dog, all doses of hydroxocobalamin were administered intravenously and doses ranged from 75 to 1200 mg/kg. There were no deaths at any dose. The major clinical signs included reddish discoloration of the skin and mucous membranes and reddish urine at all doses. Wrinkles and or wheals about the head, swollen ears, and head edema developed, possibly signs of hypersensitivity or osmotic fluid shifts with drug distribution. At the higher doses of 300 and 1200 mg/kg, emesis and tremors were observed. During a recovery period, all these signs resolved. Consistent changes in liver enzymes were reported, but it was also noted that the reddish hydroxocobalamin might interfere with colorimetric methods of analysis. Validation of these measurements was not reported. Other changes in chemistry and hematology were not of toxicological significance. There were no changes in body weight or food consumption. There were no changes in EKG parameters, heart rate or blood pressure, but the times of measurement were only at 2 hours after administration. There were no ophthalmologic examinations. The target organs were kidney, liver, bone marrow and skin. The liver of high dose dogs was characterized by edema of intrahepatic sinuses with activation of Kupffer cells, multifocal small acute necrosis, and microgranulomas. The kidney findings included multifocal tubule eosinophilic casts, focal papilla hemorrhage, multifocal tubular dilatation, and crystalline intracytoplasmic deposits in the distal tubule. In the bone marrow, there was minimal to moderate single cell necrosis that appeared to be dose-

dependent in incidence and severity. These were thought to be macrophages, but this was not verified by special staining techniques. The ratios of hematopoietic cells were not altered, but this was a subjective rather than objective measure. In the gall bladder, adrenals and fat tissue, hemorrhages were present. With time post-treatment the occurrence and severity of these findings decreased, but resolution from high doses was not complete by 8 weeks post-treatment.

Impurities of the drug product consist of



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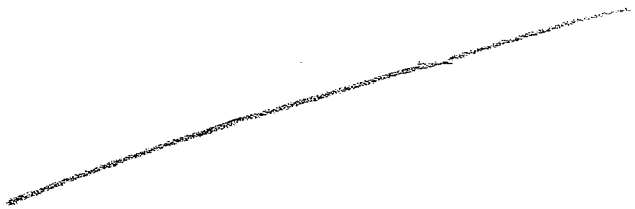

Genetic toxicology: Studies were conducted for both mutagenic and clastogenic potential of hydroxocobalamin. In reverse mutation bacteria assays, hydroxocobalamin, at doses up to 5000 µg/plate, was not mutagenic to strains of *S. typhimurium* and *E. coli* in the absence or presence of S9 mix. Hydroxocobalamin was not mutagenic at the TK locus (5-trifluorothymidine resistance) in mouse lymphoma cells using a fluctuation protocol at doses of 158 to 5000 µg/mL, with or without S9.

In the in vivo clastogenic assay, hydroxocobalamin was administered to male rats at doses up to 140 mg/kg, sufficient to induce clinical signs of toxicity, but did not induce micronuclei in rat bone marrow polychromatic erythrocytes and did not alter the ratio of

normochromic to polychromic erythrocytes. Therefore, hydroxocobalamin was not clastogenic in this assay.

Carcinogenicity: There were no carcinogenicity studies.

Reproductive toxicology: Embryo-fetal toxicity studies were conducted with rats and rabbits in 1974. These studies did not meet current GLP and ICH guidelines and therefore could not be used to support the reproductive safety of the proposed product.

Local Tolerance: There were no separate studies on local tolerance. The Sponsor did not indicate abnormal clinical or histopathological findings for injection sites in the dog toxicology studies. In the rat studies, following intraperitoneal injection, some abdominal pathological findings were attributed to the injection.

In the dog studies, hypersensitivity-like swelling of regions of the head and ears and the presence of wrinkles or wheals occurred soon after intravenous administration of hydroxocobalamin and cyanocobalamin (1 male at the high dose). These reactions resolved within a few hours or by the following day, somewhat dependent on dose administered. In one study, they reoccurred with repeated dosing on subsequent days, suggesting a fluid redistribution, rather than an immunologic hypersensitivity reaction. The cause of this reaction was not identified, since the infused solution was supposedly iso-osmotic.

Special toxicology: Hydroxocobalamin was not phototoxic to *in vitro* cultures of mouse fibroblasts at doses up to 1000 µg/mL with or without exposure to UVA light wavelengths. Toxicity measures of this assay reflect only the most serious degree, that of cell death. Phototoxicity may also cause less severe cellular reactions in skin resulting in erythema, swelling, and eschar formation, altered DNA (thymidine dimmer, adduct formation) which may lead to mutagenesis and tumors, not detectable by this assay. This assay did not test UVB wavelengths, nor does it consider other types of cells in the skin.

OVERALL CONCLUSIONS AND RECOMMENDATIONS

Conclusions:

General Toxicology: Single and repeated dose toxicological studies were performed in rats and dogs. Many of these studies did not satisfy our recommended guidelines in terms of number of animals, gender of animals, length of treatment, and parameters measured. However, in total, the major toxicities could be identified.

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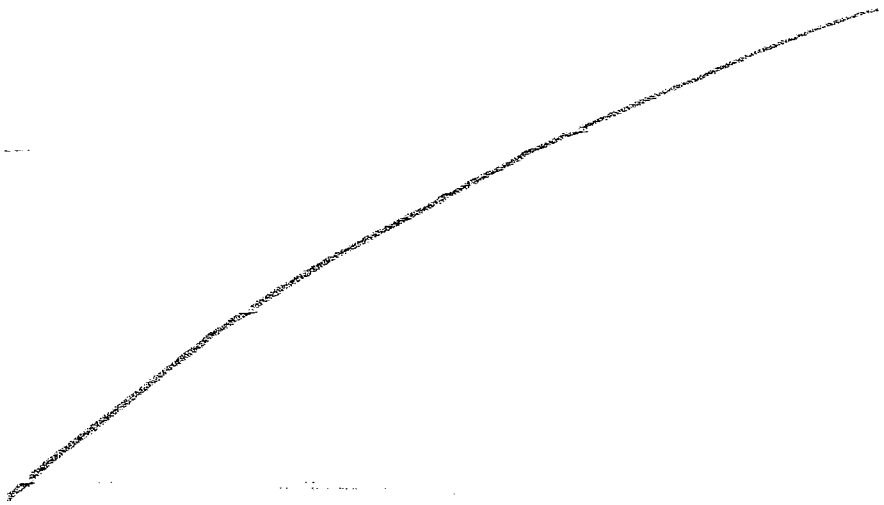

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dose administered. They were not apparent with dosing on subsequent days. The cause of this reaction was not identified.

Special toxicology: Hydroxocobalamin was not phototoxic to *in vitro* cultures of mouse fibroblasts at doses up to 1000 µg/mL with or without exposure to UVA light wavelengths. Toxicity measures of this assay reflect only the most serious degree, that of cell death. Phototoxicity may also cause less severe cellular reactions in skin resulting in erythema, swelling, and eschar formation, altered DNA (thymidine dimmer, adduct formation) which may lead to mutagenesis and tumors, not detectable by this assay. This assay did not test UVB wavelengths, nor does it consider other types of cells in the skin.

Main Efficacy Study: The main efficacy study clearly demonstrated the pharmacological efficacy of hydroxocobalamin. In anesthetized dogs, administration of a lethal dose of cyanide, followed immediately by an infusion of 75 or 150 mg/kg hydroxocobalamin resulted in dose-related survival, achieving 100% survival in dogs administered 150 mg/kg hydroxocobalamin. A dose of 75 mg/kg resulted in 79% survival. Dogs survived to 15 days post-treatment, a time at which minimal clinical or neurological signs were observed in the dogs. The ability for this agent to be infused intravenously has an important clinical advantage, in that it could be titrated to affect.

Unresolved toxicology issues

The Sponsor has not completed adequate reproduction and developmental toxicology studies for hydroxocobalamin. Since these studies are not critical to the products immediate life-saving indication, it is felt that they may be submitted as a Phase 4 Commitment. The Sponsor should conduct studies to characterize the toxicity of their product in the following conditions as described in ICHM3, S5A, S5B, and S5B(M) Guidances to Industry:

Fertility and Early Embryonic Development
Embryo-fetal Development in two species
Peri- and Post-natal Development

To support the proposed higher specification limits, genetic toxicology studies (for mutagenicity and for chromosome damage) and an acute toxicology study should be conducted with the product containing the amount of impurity at or exceeding the proposed limits, or with the isolated impurity, itself.

The toxicity of cyanocobalamin, the product of cyanide binding with hydroxocobalamin, is not well characterized, although two repeated-dose studies were conducted in dogs to address this issue. Unfortunately, they were relatively short-term and conducted with few animals. The purity of the cyanocobalamin was also not characterized. Tissue accumulation of cyanocobalamin occurred, and it was not determined how long this takes to be eliminated. In this case, single dose studies with toxicokinetics, clinical chemistry and hematology, and multiple sacrifice intervals would be useful, preferably in dogs.

Again, since these studies are not critical to the product's immediate life-saving indication, it is felt that they may be submitted as a Phase 4 Commitment.

An additional issue concerns potential phototoxicity and photosensitization. One of the target organs for drug accumulation was the skin. This was visibly detected within minutes after infusion and persisted for days depending on dose and frequency of administration. A European approved test for phototoxic effects was performed *in vitro* with fibroblast cells. Although the test was negative, this single study with only one of many cell types comprising the skin is not very conclusive as an overall determination of phototoxicity. Both hydroxocobalamin and cyanocobalamin discolor skin and organs and is not readily eliminated from tissues. Therefore, they should verify these findings with an *in vivo* phototoxicity study. This would help determine if treated patients should avoid sunlight and the length of avoidance, if necessary. Again, since these studies are not critical to the product's immediate life-saving indication, it is felt that they may be submitted as a Phase 4 Commitment

Recommendations:

Recommendation on approvability

From a nonclinical pharmacology toxicology perspective, NDA 22-041 is approvable. The reproductive and developmental toxicological information is incomplete and does not meet current ICH and FDA guidances. In addition, the recently proposed impurity limits (not in the original NDA submission) exceed the impurity concentrations that have been qualified through genotoxicity and acute toxicity studies.

Suggested labeling:

~~Red strikethrough~~ refers to deletion of the Sponsor's label
Blue refers to additions to the Sponsor's label

8 USE IN SPECIFIC POPULATIONS
8.1 Pregnancy

Pregnancy Category C.

b(4)

2 Page(s) Withheld

_____ Trade Secret / Confidential (b4)

✓ Draft Labeling (b4)

_____ Draft Labeling (b5)

_____ Deliberative Process (b5)

Withheld Track Number: Pharm/Tox- 3

APPENDIX/ATTACHMENTS

Appendix 1: Summary of Impurity Qualifications

Key Findings: Batches 9337 and 2080 with total impurities of up to % are qualified. The qualifying studies were not ideally performed, but the results are consistent with studies with lower impurity batches. The single dose animal study was performed at with only female rats, but the results were similar to studies performed with both males and females with lower impurity batches. There were no obvious differences between genders in those studies, but since more females died, it would suggest that female rats would be more sensitive than male rats. The genetic toxicology studies only comprised a mutagenic test; a test for clastogenic potential was not conducted. The bacterial mutagenic (Ames) test was negative for mutagenic potential. All of the genotoxic studies, tests for both mutagenic and clastogenic potential, with batches containing less impurities were negative.

Drug Batches used in stability studies, then used for impurity testing.

Batch: 9337: stored at 25°C/60% RH for 36 months
Batch 2080: stored at 40°C/75% RH for 8 months

Genetic Toxicology
(See Genetic Toxicity Table, below)
Two genotoxicity studies are recommended, usually a test of Mutagenic potential (bacteria) and a test of Clastogenic potential (*in vitro* or *in vivo* micronucleus). However, only one of the two assays, the bacteria mutagenic assay, was performed with batches of hydroxocobalamin that contained high concentrations of impurities. This was negative for mutagenic potential.

Reviewer's Comment's: A second genotoxic test, a clastogenicity assay, should be performed with both batches, or at least Batch 2080 which has the highest concentration of impurities.

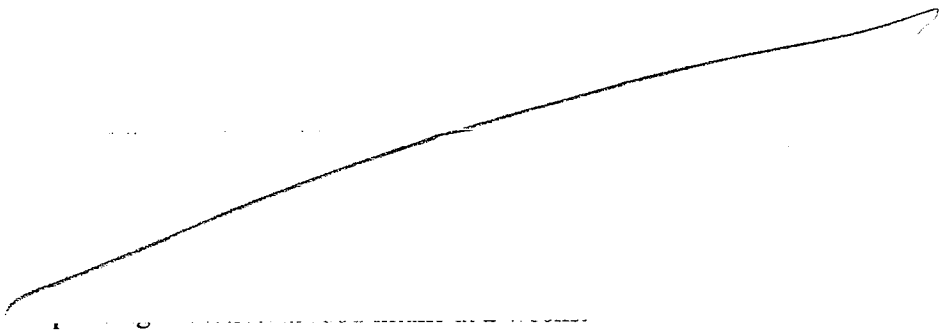
Acute Toxicology
(See Toxicology Studies in Rat Table, below)
A single dose administration followed by 14 days of observation was conducted in rats with similar doses and methods for both Batches of hydroxocobalamin that contained high concentrations of impurities. Toxicological results were similar with both Batches.

These results were compared with results from batches that contained lower concentrations of impurities. Toxicological results were similar for Batches with or without high concentrations of impurities.

Reviewer's Comment's: Cautionary note: in these rat studies, all doses appeared to produce close to similar effects, as if dosing was excessive, and tissues were saturated.

While this may be useful in identifying toxicity, it may mask more subtle toxicological differences that may be seen at lower doses.

Main Efficacy Study: Dog
(see Toxicology Studies in Dog Table, below)



b(4)

Conclusion: the higher ——— concentration in the Dog Efficacy Study probably did not contribute any additional toxic responses over those already characterized for hydroxocobalamin.

Also, there was a much lower concentration of substance with a ———, ...%, than in Batches used in previous dog studies (—% and —%) or any of the rat studies (———% and —%). b(4)

Conclusion: with a lower concentration of substance with a ———, there was no obvious differences in responses compared to those already characterized with a higher concentration of this substance.

APPEARS THIS WAY
ON ORIGINAL

Impurity Qualification: Genetic Toxicology Studies (Reviewer created table)

Batch	**Release Specs (%)**	**Shelf-life Specs (%)**	**2056**	**2070**	**9337**	**2080**	**2056**	**2056**
Manufacturing Date			Aug 30, 2002	Dec 12, 2003	Aug 30, 2000	July 30, 2004	Aug 30, 2002	Aug 30, 2002
Release Date (if stated in study)					Jan 29, 2001			
Stability testing					**stored at 25°C/60% RH for 36 months**	**stored at 40°C/75% RH for 8 months**		
Analysis Date			Oct 4, 2002, Dec 15, 2003 (Retest with proposed HPLC method)	March 31, 2004 (Retest with the proposed HPLC method)	March 31, 2004 (Retest with the proposed HPLC method) this is 2 months after the end of 36 months of stability testing (43 months after manufactured)	Aug 3, 2005 (Retest with the proposed HPLC method) (this is 12 months after manufactured)	Oct 4, 2002, Dec 15, 2003 (retest with proposed HPLC method)	Oct 4, 2002, Dec 15, 2003 (retest with proposed HPLC method)
Other unspecified impurities								
Total impurities								

b(4)

Batch			**2056**	**2070**	**9337**	**2080**	**2056**	**2056**
Study			**T15570** Bacterial Mutagenicity Assay	**T15950** Bacterial Mutagenicity Assay	**T15917** Bacterial Mutagenicity Assay	**T16401** Bacterial Mutagenicity Assay	**T15574** *In Vivo* Rat Micronucleus Assay	**T15575** *In Vitro* Gene Mutation Assay (L5178Y/TK+/-)
First use of drug in the study			March 13, 2003	Dec 14, 2004 (Dec 1, 2004)	Nov 23, 2004	Oct 19, 2005	April 15, 2003	May 12, 2003
Time interval (manufacture to use)			5.5 months	12 months	51 months	14.5 months	6.5 months	7.5 months
Study Toxicology Findings			**Negative**	**Negative**	**Negative**	**Negative**	**Negative**	**Negative**

Impurity Qualification: Toxicology Studies in Rat (Reviewer created table)

Batch	Release Specs (%)	Shelf-life Specs (%)	2056	2059	9337	2080
Note: Batch 2056 toxicological findings were used to enable comparison (bridging) of rat toxicology findings in high impurity batch studies, 9937 and 2080, with batch 2081 used in the dog efficacy study, since batch 2056 and the dog efficacy batch 2081 had closely similar impurity concentrations.						
Manufacturing Date			Aug 30, 2002	Feb 20, 2003	Aug 30, 2000	July 30, 2004
Release Date (stated in study)					Jan 29, 2001	
used for Stability testing					stored at 25°C/60% RH for 36 months	stored at 40°C/75% RH for 8 months
Analysis Date			Oct 4, 2002, Dec 15, 2003 (retest with proposed HPLC method)	March 21, 2003 at release with previous PL method Dec 15, 2003 (retest with proposed HPLC method)	March 31, 2004 (Retest with the proposed HPLC method) (this is 2 months after the end of 36 months of stability testing (this 43 months since manufactured)	Aug 3, 2005 (Retest with the proposed HPLC method) (this is 12 months since manufactured)
Other unspecified impurities						
Total impurities						
Batch			**2056**	**2059**	**9337**	**2080**
Study			**T15741** single dose 300, 600, 800, 1000 mg/kg, ip	**T15096** TK study 300 mg/kg, ip.	**T151948** single dose 300, 600,1000 mg/kg, ip	**T16400** single dose 300, 600, 1000 mg/kg, ip
First use of drug in the study			Nov 18, 2003	July 8, 2005	Nov 12, 2004	Oct 17, 2005
Time interval (manufacture to use)			analysis was 1 month after the study was initiated (14 months since manufactured)		this 8 months after analysis (51 months since manufactured	this is 2.5 months after analysis (14.5 months since manufactured)

b(4)

Batch			2056	2059	9337	2080
Study			T15741	T15096	T151948	T16400
Toxicology Findings	(see specific studies for further details)					
			Female results only presented		**only Females studied**	**only Females studied**
	Deaths		**1/3 Death at 600 mg/kg 3/3 Deaths at 1000 mg/kg**	**none**	**2/3 Deaths at 1000 mg/kg;**	**2/3 Deaths at 1000 mg/kg;**
	Clinical Signs		up to 6 days after dosing	No clinical signs noted	up to 7 days after dosing	up to 7 days after dosing
	dyspnea		+		+	+
	skin reddened		+		+	+
	piloerection		+		+	+
	locomotor disturbance		+		+	+
	abdominal position		+		+	+
	sunken flanks		+		+	+
	incomplete eyelid closure		+		+	+
	reddish urine		+		+	+
	feces retention		+		+	+
	Pathology					
			red discoloration of peritoneum and skin		red discolored body,	reddish discoloration of all organs and tissues,
			reddish fluid in thoracic cavity and abdominal cavity		red fluid in thoracic cavity	
			blood congestion in liver and lung		abdominal adhesions	multifocal adhesions,
			dilation of small intestine with fluid		focal and multifocal white nodules (foreign body granulomas) in abdominal cavity	white particles in abdominal cavity
			focal fat necrosis		brown fat atrophy	mild focal fat necrosis with mixed inflammation

Impurity Qualification: Toxicology Studies in Dog (Reviewer created table)

Batch	Release Specs (%)	Shelf-life Specs (%)	2081 Dog Efficacy Study	2056	2059	2066
Manufacturing Date			Aug 4, 2004	Aug 30, 2002	Feb 20, 2003	Oct 14, 2003
Analysis Date			Aug 27, 2004 (Release with proposed HPLC method)	Oct 4, 2002, Dec 15, 2003 (retest with proposed HPLC method)	March 21, 2003 Dec 15, 2003 (retest with proposed HPLC method)	Nov 13, 2003 March 31, 2004 (retest with proposed HPLC method)
Other unspecified impurities **Total impurities**						
Study			**N-106342** main study **(N-106341** pilot study)	**T8347** 14-day repeated dosing 150, 300, 600 mg/kg, iv.	**T8348** 4-week repeated dosing with 8 week recovery 75, 150, 300 mg/kg, iv	**T8374** Single dose 150, 300, 1200 mg/kg, iv. **T8355** 3-day repeated dose 300, 600, 1200 mg/kg,iv.
First use of drug in the study			Nov 26, 2004 (March 16, 2005)	Feb 3, 2003	May 19, 2003	Nov 1, 2004 (Study T8374)
Time interval (manufacture to use)			3 months after analysis (4 months since manufactured)	5 months	3 months	12.5 months

b(4)

Appendix 2: Literature References

Study Number/Location	Authors/Title/Citation
4.3.1 Vol. 13, Tab 4.3.1, Astier 1996	Astier A, Baud FJ. Complexation of Intracellular Cyanide by Hydroxocobalamin Using a Human Cellular Model. Human and Experimental Toxicol. 1996;15:19-25
4.3.2 Vol. 13, Tab 4.3.2, Astier 1995	Astier A, Baud FJ. Simultaneous Determination of OHCo and its Cyanide Complex CNCo in Human Plasma by HPLC Application to PK Studies after High Dose OHCo as an Antidote for Severe Cyanide Poisoning J Chromatogr B 1995;667: 129-35
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I concur.