2.6.6.4 Genetic toxicology

1) Evaluation of PDX in the Salmonella typhimurium/Escherichia coli Plate Incorporation Mutation Assay in the Presence and Absence of Induced Rat Liver S-9

Major finding

Under the conditions of this study, pralatrexate caused no increase in the incidence of reverse mutation in the *Salmonella Typhimurium* or *Escherichia coli* strains with and without metabolic activation.

Study no: Sponsor – PDX-T-06021-I; Laboratory – 0902-2140
File name: pdxt06021i—nonclinical-data.pdf
Laboratory: (b) (4)
Study Date: September 2005
GLP: Yes
Audited: Yes
Drug: Pralatrexate Injection, Lot 00510105, Purity 98.5 %
Method:
Test Strains: Salmonella typhimurium strains TA98, TA100, TA1535 and TA1537, and Escherichia coli strain WP2 uvrA
Range Finding: 5.0, 10, 50, 100, 500, 100, 5000 µg/plate
Mutation Doses: 100, 500, 1000, 3000 or 5000 µg/plate (in triplicate)
Positive Controls:

Strain	Code number	S9	Chemical	Conccentration µg/plate
TA98	1	-	2-NF (2-Nitrofluorene)	5
TA98		+	2-AA (2-Aminoanthracene)	2.5
TA100	2	-	NaAz (Sodium Azide)	1
TA100		+	2-AA (2-Aminoanthracene)	2.5
TA1535	3	-	NaAz (Sodium Azide)	1
TA1535		+	2-AA (2-Aminoanthracene)	2.5
TA1537	4	-	9-AA (9-Aminoacridine)	75
TA1537		+	2-AA (2-Aminoanthracene)	5
WP2 uvrA	5	-	MMS (Methyl Methanesulfonate)	4000
WP2 uvrA		+	2-AA (2-Aminoanthracene)	20

Negative Control: PBS and DMSO
Metabolic Act. Aroclor 1254 induced rat liver microsomes, S9

Results:

Range finding: There were no signs of toxicity or precipitation in any of the test strains at concentrations to 5000 µg/plate.
Mutation Assay 1: There was no increase in reverse mutations in any of the strains and no signs of toxicity or precipitation at concentrations to 5000 µg/plate
Mutation Assay 2: There was no increase in reverse mutations in any of the strains and no signs of toxicity or precipitation at concentrations to 5000 µg/plate
Positive Controls: All positive controls caused a significant and consistent increase in reverse mutations

The following table from the submission shows the summary results from Mutation Assay 1 in all strains without activation.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
MUTATION ASSAY RESULTS - WITHOUT ACTIVATION

SPONSOR: Allos Therapeutics, Inc.
EXPERIMENT NO.: B-1
TEST ARTICLE: PDX(Pralatrexate)

(b) STUDY NO.: 0902-2140
SOLVENT: PBS
CONC. IN: µg/plate

S. typhimurium		Average No. of Revertants Per Plate						
		Positive Control	Solvent Control	Concentration per plate 100	500	1000	3000	5000
STRAIN: TA98	REVERTANTS	678	39	37	36	35	25	28
DATE PLATED:	STD. DEV.	60	3	4	2	9	11	7
10/18/05	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 7.540E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	534	75	67	74	78	62	69
DATE PLATED:	STD. DEV.	32	14	10	6	1	9	12
10/18/05	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 7.580E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	765	40	46	52	48	50	39
DATE PLATED:	STD. DEV.	60	3	8	3	5	2	11
10/18/05	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 6.100E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	272	12	8	8	8	7	10
DATE PLATED:	STD. DEV.	30	3	4	2	2	1	5
10/18/05	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 5.640E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 100	500	1000	3000	5000
STRAIN: WP2 uvrA	REVERTANTS	478	17	14	16	18	13	14
DATE PLATED:	STD. DEV.	35	4	2	2	3	3	2
10/18/05	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.138E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

The following table from the submission shows the summary results from Mutation Assay 1 in all strains with activation.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
MUTATION ASSAY RESULTS - WITH S-9 ACTIVATION

SPONSOR: Allos Therapeutics, Inc.
EXPERIMENT NO.: B-1
TEST ARTICLE: PDX(Pralatrexate)

(b) STUDY NO.: 0902-2140
SOLVENT: PBS
CONC. IN: µg/plate

S. typhimurium		Average No. of Revertants Per Plate						
		Positive Control	Solvent Control	Concentration per plate 100	500	1000	3000	5000
STRAIN: TA98	REVERTANTS	1248	37	41	36	41	37	38
DATE PLATED:	STD. DEV.	36	4	7	5	5	4	6
10/18/05	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 7.540E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	1146	62	63	69	71	81	66
DATE PLATED:	STD. DEV.	72	0	9	2	14	6	6
10/18/05	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 7.580E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	248	14	12	15	19	16	14
DATE PLATED:	STD. DEV.	17	2	4	2	3	6	6
10/18/05	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 6.100E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	362	14	11	14	15	10	13
DATE PLATED:	STD. DEV.	17	2	5	2	3	3	3
10/18/05	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 5.640E+07	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 100	500	1000	3000	5000
STRAIN: WP2 uvrA	REVERTANTS	276	15	13	15	18	19	19
DATE PLATED:	STD. DEV.	16	3	5	5	2	5	2
10/18/05	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.138E+08	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

---

2) Test for Chemical Induction of Chromosome Aberrations in Cultured Chinese Hamster Ovary (CHO) cells with and without Metabolic Activation

Major finding

Pralatrexate was cytotoxic at concentrations above 10 µM. At lower concentrations the compound caused a dose dependant decrease in relative mitotic index (RMI) but the cells maintained a normal morphology. In the absence of metabolic activation, the IC_{50} was 1.4 µM for CHO cells in culture (3 hour exposure) based on RMI. The addition of S9 increased the IC_{50} to 4.3 µM. With an 18 hour exposure, the compound did not prevent DNA synthesis as evidenced by the incorporation of BrdU within growing cells at concentrations less than or equal to 0.21 µM. The compound did not cause an increase in chromosomal aberrations at concentrations between 0.5 and 2.1 µM in the absence or presence of S9. After exposure for 18 hours to concentrations between 0.0063 and 0.027 µM, pralatrexate caused no apparent increase in the percentage of cells with aberrations.

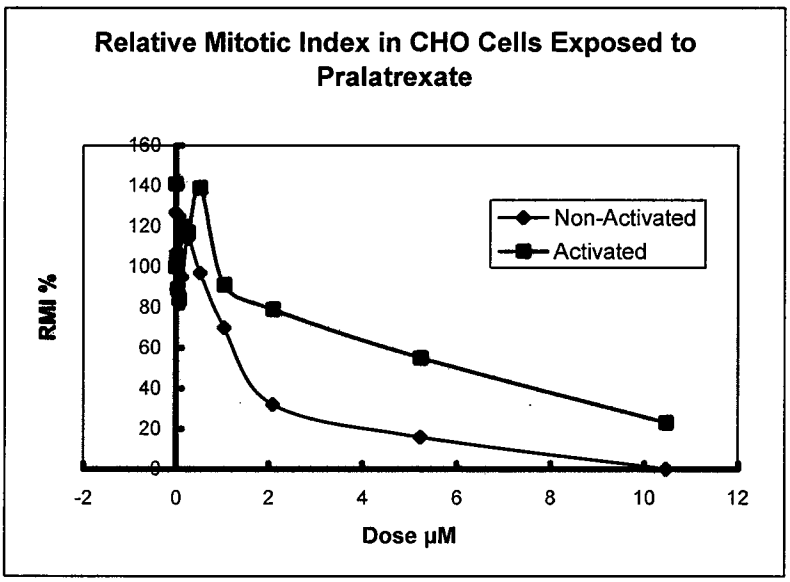
Nevertheless, there were significant problems with this study. The results from three assays were absent from the report and the highest concentrations evaluated in the presence of metabolic activation were lower than the IC_{50} in some cases.

Study no:	Sponsor – PDX-T-06022-H; Laboratory – 0902-3110
File name:	pdxt06022-H—nonclinical-data.pdf
Laboratory:	(b) (4)
Study Date:	September 2005
GLP:	Yes
Audited:	Yes
Drug:	Pralatrexate Injection, Lot 00510105, Purity 98.5 %
Method:	
Cells:	Chinese Hamster Ovary Cells (CHO)
Range Finding:	Test A-1 – 5.0, 10, 50, 100, 500, 1000, 2500, and 5000 µg/mL 0.01, 0.021, 0.1, 0.21, 1.05, 2.09, 5.24, 10.5 mM Not scored for aberrations Test A-2 – 0.008, 0.016, 0.031, 0.063, 0.125, 0.025, 0.5, 1.0, 2.5, and 5 µg/mL, Not scored 0.017, 0.034, 0.065, 0.132, 0.262, 0.524, 1.05, 2.09, 5.24 and 10.47 µM
Mutation Doses:	Test B-1 – 0.25, 0.5, 1.0, and 2.5 µg/mL in activated systems 0.125, 0.25, 0.5, and 1.0 µg/mL in non-activated systems, Scored for 0.25, 0.5 and 1.0 µg/mL for both Test B-2 – 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 µg/mL without activation only Not scored BrdU Labeling assay – 0.0002 – 0.1 µg/mL 500 cells scored per slide Test B-3 – 0.0002 to 0.1 µg/mL Scored for 0.003, 0.006 and 0.013 Test B-4 – 0.25, 0.5, 1.0, and 2.5 µg/mL activated system 0.125, 0.25, 0.5, and 1.0 non-activated system Test B-5 – 0.025, 0.0125, 0.00625, 0.00313, 0.00156, and 0.00078 µg/mL in non-activated system only
Solvent	PBS
Treatment time	Three hours drug exposure, 18 hours incubation (total) with Colchemid added in the last two hours for A-1, A-2, B-1, B-4 18 hours drug exposure (18 hours total incubation) with Colchemid added the last two hours for B-5 18 hours B3 (no Colchemid)
Replicates	Two
Positive Controls:	Mitomycin-C (MMC) in the absence of activation Cyclophosphamide (CP) in the presence of metabolic activation
Metabolic Act.	phenobarbital/ß-naphthoflavone-induced rat liver homogenate (S9)
Analysis	The dosing solutions were sent to a laboratory at the University of Iowa to determine their concentration by HPLC

Results:

Solubility –	Pralatrexate was soluble at all concentrations up to 5000 µg/mL
Test A1 –	Pralatrexate was toxic at all concentrations in this assay

Test A2 – Both mitotic index and RCG (relative cell growth) decrease with increasing concentration. The IC_{50} based on mitotic index (calculated in GraphPad, sigmoid dose response model) for the non-activated experiment is 1.4 μM (0.65 μg/mL, $r^2 = 0.94$); for the activated experiment it was 4.3 μM (2.1 μg/mL, $r^2 = 0.76$). In the presence of S9 the RMI was 55% at a concentration of 5.2 μM (2.5 mg/mL). This fact and the IC_{50} indicate that this concentration and at least two lower should probably be scored. In both activated and non-activated systems, "cell morphology was normal and healthy as observed under the microscope."



Test B-1 According to the narrative on page 17 in the Procedures section, this experiment was completed and the cells in flasks containing 0.25, 0.5 and 1 μg/mL were scored. Since the highest concentration is greater than the IC_{50} for mitotic index this scoring would be adequate. Nevertheless, the narrative on page 21 in the Procedures section states "The definitive (B-1) and confirmatory (B-3) Chromosome Aberration Assays were aborted since the dosing solutions were unable to be analyzed due to leakage during shipment." It is not clear from the report exactly which samples this sentence refers to but it is likely that they are samples sent to a laboratory at the (b) (4) for concentration analysis. The study report does not contain results for Test B1 or B3.

Test B-2 According to the narrative on page 19 "A confirmatory Chromosome Aberration Assay (B-2) was performed without activation only, because the results from the definitive assay (B-1) were negative in the without activated system. The concentrations tested were 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/mL. ..... Chromosome aberrations from the Confirmatory Assay (B-2) were not scored because mitotic index was very low (mitotic index 1% at lowest dose tested). The morphology of cells harvested at all concentrations in the B-2 assay was healthy which indicated possible cytostatic nature of pralatrexate. In order to sort out whether test article

inhibited DNA synthesis, a mechanistic study using BrdU (thymidine analogue) labeling technique was performed." The study report does not present these results except for these comments in the narrative.

BrdU Label assay — BrdU labeled more than 99% of the cells in the pralatrexate treated cultures. Thus, the pralatrexate did not inhibit DNA synthesis. The study report does not present these results except in the narrative.

Test B-4 — The following table from the study report shows that Relative Mitotic Index (RCG) decreased with increasing drug concentration.

TABLE 4
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
MITOTIC INDEX - DEFINITIVE ASSAY

SPONSOR : Allos Therapeutics, Inc. SOLVENT : PBS STUDY NO. : 0902-3110
TEST ARTICLE : PDX (Pralatrexate) TRIAL NO.: B4

Without Activation - Treatment: 3 Hours Harvest: 18 Hours						With Activation - Treatment: 3 Hours Harvest: 18 Hours					
Test Article Concentration (µg/mL)	Tube No.		No. of Dividing Cells/500	Mean Mitotic Index	Relative Mitotic Index	Test Article Concentration (µg/mL)	Tube No.		No. of Dividing Cells/500	Mean Mitotic Index	Relative Mitotic Index
Untreated	92	A	55	10.9	99%	Untreated	7	A	59	11.2	114%
		B	54					B	53		
Solvent	49	A	60	11.0	100%	Solvent	70	A	53	9.8	100%
		B	50					B	45		
0.125	48	A	64	10.8	98%	0.25	13	A	45	9.9	101%
		B	44					B	54		
0.25	12	A	60	11.2	102%	0.5	97	A	41	9.8	100%
		B	52					B	57		
0.5	45	A	21	5.1	46%	1.0	65	A	32	4.9	50%
		B	30					B	17		
1.0	51	A	18	3.8	35%	2.5	52	A	20	3.3	34%
		B	20					B	13		
MMC 0.4	19	A	38	7.1	65%	CP7.5	54	A	12	2.8	25%
		B	33					B	16		
MMC 0.8	47	A	28	5.2	48%	CP12.5	41	A	7	1.2	11%
		B	24					B	5		

All test article concentrations were compared to solvent.
The positive controls were compared to Untreated since the solvent for MMC and CP was water.

$$MI = \frac{\text{No. of dividing cells scored from 1000 cells}}{10} \qquad RMI = \frac{\text{Test Dose MI}}{\text{Solvent Control MI}} \times 100$$

The following tables from the study report show that the frequency of chromosomal aberrations did not increase significantly with increasing dose in the presence or absence of metabolic activation. The concentration scored without metabolic activation, 1.0 µg/mL, is greater than the IC_{50} determined in the range finding study, A2, so the scoring is adequate. In the test with metabolic activation, the highest concentration scored is below the IC_{50} determined in test A2 but the finding of a 34% decrease in mitotic index (above) suggests that scoring is adequate. While within the bounds of traditional procedure, the results of scoring in the high dose group, 2.5-µg/mL, would have been informative.

TABLE 5
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
CHROMOSOME ABERRATIONS - DEFINITIVE ASSAY

TEST ARTICLE: PDX(Pralatrexate)
SPONSOR: Allos Therapeutics, Inc.
SOLVENT: PBS

TREATMENT TIME: 3 Hours
HARVEST TIME: 18 Hours

(b) STUDY NO.: 0902-3110
TRIAL NO.: B4
METABOLIC ACTIVATION: Yes ( ) No (X)

TREATMENT AND CONC. (µg/mL)	CELLS Scored	NUMBER AND TYPE OF ABERRATIONS																	NO. OF ABS. PER CELL	% CELLS WITH ABS.	P-VALUE IN CHI-SQUARE**
		NOT COMPUTED				Chromatid Type							Chromosome Type								
						Simple		Complex					Simple	Complex			Others				
		tg	sg	% e	%pp	tb	isb	tr	qr	cr	id	ci	sb	d	r	dm	pu	sd*			
Untreated A	100			0	0														0.00	0.0	
Untreated B	100			0	2														0.00	0.0	
Untreated A+B	200			0.0	1.0														0.000	0.0	
Solvent A	100			0	0														0.00	0.0	
Solvent B	100			0	2														0.00	0.0	
Solvent A+B	200			0.0	1.0														0.000	0.0	
0.25 A	100			1	0														0.00	0.0	
0.25 B	100			0	1														0.00	0.0	
0.25 A+B	200			0.5	0.5														0.000	0.0	=Solvent
0.5 A	100	2		0	0														0.00	0.0	
0.5 B	100			0	0														0.00	0.0	
0.5 A+B	200	2		0.0	0.0														0.000	0.0	=Solvent
1.0 A	100		1	0	0														0.00	0.0	
1.0 B	100			1	2	1													0.01	1.0	
1.0 A+B	200		1	0.5	1.0	1													0.005	0.5	≤1***
MMC 0.4 A	100			1	0	5	3	11	9	2		1	5						0.36	27.0	
MMC 0.4 B	100	2		0	0	16		4	8		1		10		3				0.42	33.0	
MMC 0.4 A+B	200	2		0.5	0.0	21	3	15	17	2	1	1	15		3				0.390	30.0	≤0.001

* sd = 10 aberrations in calculations.

**Statistical analyses done on the % cells with aberrations . It was considered significant if $p \leq 0.05$.

In Chi-square test, all test article concentrations compared to (b) historical solvent(water) control data(0.22%) since solvent for the test article is PBS and concurrent value is 0%.

In Chi-square test MMC compared t (b) s historical Untreated control data (0.22%) since solvent for MMC was water and concurrent value is 0%.

***The distribution was not significant.

TABLE 6
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
CHROMOSOME ABERRATIONS - DEFINITIVE ASSAY

TEST ARTICLE: PDX (Pralatrexate) TREATMENT TIME: 3 Hours (b) JDY NO.: 0902-3110
SPONSOR: Allos Therapeutics, Inc. HARVEST TIME: 18 Hours TRIAL NO.: B4
SOLVENT: PBS METABOLIC ACTIVATION: Yes (X) No ( )

TREATMENT AND CONC. (µg/mL)	CELLS Scored	NUMBER AND TYPE OF ABERRATIONS																	NO. OF ABS. PER CELL	% CELLS WITH ABS.	P-VALUE IN CHI-SQUARE**
		NOT COMPUTED				Chromatid Type							Chromosome Type				Others				
						Simple		Complex					Simple	Complex							
		tg	sg	% e	%pp	tb	isb	tr	qr	cr	id	ci	sb	d	r	dm	pu	sd*			
Untreated A	100			0	0														0.00	0.0	
Untreated B	100			0	3														0.00	0.0	
Untreated A+B	200			0.0	1.5														0.000	0.0	
Solvent A	100			0	1														0.00	0.0	
Solvent B	100			0	2														0.00	0.0	
Solvent A+B	200			0.0	1.5														0.000	0.0	
0.25 A	100			0	0														0.00	0.0	
0.25 B	100			0	3														0.00	0.0	
0.25 A+B	200			0.0	1.5														0.000	0.0	=Solvent
0.5 A	100			1	1														0.00	0.0	
0.5 B	100			0	1														0.00	0.0	
0.5 A+B	200			0.5	1.0														0.000	0.0	=Solvent
1.0 A	100			0	0														0.00	0.0	
1.0 B	100	1		3	2	1													0.01	1.0	
1.0 A+B	200	1		1.5	1.0	1													0.005	0.5	≤1***
CP 7.5 A	100			0	0	8	10	5	2	2	6	3	13						0.49	29.0	
CP 7.5 B	100			0	0	13		9	10		1		4		2				0.39	30.0	
CP 7.5 A+B	200			0.0	0.0	21	10	14	12	2	7	3	17		2				0.440	29.5	≤0.001

* sd = 10 aberrations in calculations.

**Statistical analyses done on the % cells with aberrations. It was considered significant if $P \leq 0.05$.
In Chi-square test, 1.0µg/mL test article concentration compared (b) (4) treated(water) control data(0.37%) since solvent for test article is PBS and concurrent value is 0%.
In Chi-square test CP compared to (b) historical Untreated control data (0.37%) since solvent for CP was water and concurrent value is 0%.
***The distribution was not significant.

Test B-5

The following table from the study report shows the decrease in mitotic index with increasing concentrations after incubation with pralatrexate for 18 hours without metabolic activation. The IC_{50} for mitotic index calculated from this data is 0.0073 µg/mL (estimated in GraphPad using the sigmoid dose response model, $r^2 = 0.84$). The scoring that follows is adequate.

TABLE 8
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
MITOTIC INDEX - CONFIRMATORY ASSAY

SPONSOR : Allos Therapeutics, Inc. SOLVENT : PBS STUDY NO. : 0902-3110
TEST ARTICLE : PDX(PRALATREXATE) TRIAL NO.: B5

Without Activation - Treatment: 18 Hours Harvest: 18 Hours						With Activation
Test Article Concentration (ug/mL)	Tube No.		No. of Dividing Cells/500	Mean Mitotic Index	Relative Mitotic Index	
Untreated	59	A	54	10.4	118%	Activated System was not performed in B3
		B	50			
Solvent	68	A	50	8.8	100%	
		B	38			
0.00078	89	A	39	9.0	102%	
		B	51			
0.00156	40	A	41	8.1	92%	
		B	40			
0.003125	64	A	31	8.0	91%	
		B	49			
0.00625	1	A	22	5.8	66%	
		B	36			
0.0125	38	A	19	3.3	38%	
		B	14			
0.025	27	A	9	2.1	24%	
		B	12			
MMC0.2	37	A	21	3.9	36%	
		B	18			
MMC0.4	69	A	18	3.4	33%	
		B	16			

All test article concentrations were compared to solvent.
The positive controls were compared to Untreated since the solvent for MMC was water.

$MI = \frac{\text{No. of dividing cells scored from 1000 cells}}{10}$ $RMI = \frac{\text{Test Dose MI}}{\text{Solvent Control MI}} \times 100$

The following table shows that the frequency of chromosomal aberrations did not increase with increasing dose in the absence of metabolic activation in this confirmatory assay (18 hours incubation with pralatrexate).

TABLE 9
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
CHROMOSOME ABERRATIONS - CONFIRMATORY ASSAY

TEST ARTICLE: PDX(Pralatrexate) TREATMENT TIME: 18 Hours (b)1 STUDY NO.: 0902-3110
SPONSOR: Allos Therapeutics, Inc. HARVEST TIME: 18 Hours TRIAL NO.: B5
SOLVENT: PBS METABOLIC ACTIVATION: Yes ( ) No (X)

TREATMENT AND CONC. (µg/mL)	CELLS Scored	NUMBER AND TYPE OF ABERRATIONS																	NO. OF ABS. PER CELL	% CELLS WITH ABS.	P-VALUE IN CHI-SQUARE**
		NOT COMPUTED				Chromatid Type							Chromosome Type								
						Simple		Complex					Simple	Complex			Others				
		tg	sg	% c	%pp	tb	isb	tr	qr	cr	id	ci	sb	d	r	dm	pu	sd*			
Untreated A	100			0	1														0.00	0.0	
Untreated B	100			0	4								1						0.01	1.0	
Untreated A+B	200			0.0	2.5								1						0.005	0.5	
Solvent A	100			0	0	1													0.01	1.0	
Solvent B	100			0	4														0.00	0.0	
Solvent A+B	200			0.0	2.0	1													0.005	0.5	
0.003125A	100	1	2	0	0														0.00	0.0	
0.003125 B	100			0	1	2													0.02	2.0	
0.003125 A+B	200	1	2	0.0	0.5	2													0.010	1.0	≤ 1***
0.00625 A	100	1		0	0														0.00	0.0	
0.00625 B	100			0	1														0.00	0.0	
0.00625 A+B	200	1		0.0	0.5														0.000	0.0	<solvent
0.0125 A	100			0	0														0.00	0.0	
0.0125 B	100			0	1	1													0.01	1.0	
0.0125 A+B	200			0.0	0.5	1													0.005	0.5	=solvent
MMC 0.2 A	100	1	1	0	0	5	6	4	7	13	5	3	7						0.50	32.0	
MMC 0.2 B	100	1		0	0	12		8	10			2	7		3				0.42	33.0	
MMC 0.2 A+B	200	2	1	0.0	0.0	17	6	12	17	13	5	5	14		3				0.460	32.5	≤0.001

* sd = 10 aberrations in calculations.

**Statistical analyses done on the % cells with aberrations. It was considered significant if $p \leq 0.05$.

*** The distribution was not significant.

In Chi-square test, all test article concentrations were compared to solvent control data.

In Chi-square test MMC compared to Untreated control data since solvent for MMC was water.

---

3) *In Vivo* Test for Chemical Induction of Micronucleated Polychromatic Erythrocytes in Mouse Bone Marrow Cells

Major findings

Doses above 25 mg/kg (75 mg/m^2) daily for three days caused almost complete myelosuppression; these doses were too high for this micronucleus test. Lower doses at the same schedule of 0.125 to 5 mg/kg (0.375 to 15 mg/m^2) caused a dose dependant decrease in the percent of polychromatic erythrocytes (PCEs) with an IC_{50} of about 3 mg/kg (9 mg/m^2) in both males and females. These doses caused only minor systemic toxicity evidenced by mild weight loss over 72 hours. Doses of 3 mg/kg did not cause an increase in micronucleated PCE in either males or females,

There were significant problems with this study. The results from an assay were absent from the report. The values for remaining PCEs for 4 and 5 mg/kg are about 30% or above and would have been adequate for scoring. Given that this drug class is known to be genotoxic, including these two dose groups in the main test would have been informative.

Study number: Sponsor - PDX-T-06023-M, Laboratory - 0902-1521

File name:	PDX-T-0g023-M.pdf
Laboratory:	(b) (4)
Study Date:	September 2005
GLP:	Yes
Audited:	Yes
Drug:	Pralatrexate Injection, Lot 00510105, Purity 98.5 %
Method:	
Species:	male and female CD-1 mice
Range finding:	Test A1 – doses of 5, 25, 50, 100, 200, and 394 mg/kg Three animals per sex per group
Main Study:	Test B1 – doses of 100, 200 and 394 mg/kg Five mice per sex per group
Range finding:	Test A2 – doses of 5, 4, 3, 2, 1, 0.5, 0.25, and 0.125 mg/kg Test A3 – doses of 5, 4, 3, 2, 1, 0.5, 0.25, and 0.125 mg/kg Three mice per sex per group, monitored for 14 days to assess delayed toxicity
Main Study:	Test B2 – doses of 1.0, 3.0, and 5.0 mg/kg Five mice per sex per group
Main Study:	Test B3 – doses of 0.5, 1.0, 2.0, and 3.0 mg/kg
Route:	IP
Schedule:	Daily for three days
Dose volume	20 mL/kg for A1 and B1, 10 mL/kg for A2, A3, B2 and B3
Positive control:	Cyclophosphamide 80 mg/kg PO gavage (single dose)
Marrow Collection:	24 hours after the last injection
Analysis:	Two thousand PCEs per animal were analyzed for the frequency of micronuclei

Results:

Mortality	None
Test A1	The weight of HD males decreased 9.4 % whilst that of HD females decreased 6.7 % in HD females at 72 hours after the start of the experiment. All groups dosed with more than 25 mg/kg lost weight.
Test A2	The weight of HD males decreased 3% % whilst that of HD females decreased 9.7 % in HD females at 72 hours after the start of the experiment. The following table shows the decrease in the percentage of PCEs in this experiment. The IC_{50} for males and females is about 3 mg/kg (estimated with GraphPad, sigmoidal dose response model, r^2 = 0.85 for females and 0.38)

dose mg/kg	Percent PCE Male	% change in PCE Male	Percent PCE Female	% change in PCE Female
0	68.5	0	72.7	0
0.125	70.7	3%	65.3	-10%
0.25	58.2	-15%	53.3	-27%
0.5	39.3	-43%	55.3	-24%
1	52.8	-23%	51.8	-29%
2	53.6	-22%	58.7	-19%
3	36.8	-46%	46	-37%
4	20.5	-70%	36.5	-50%
5	29	-58%	35.2	-52%

Test A3

Mice gained weight in all dose groups between day 0 and day 14. This group was dosed for the purpose of monitoring delayed toxicity. There was none.

Test B1

On page 6 of the study report the text says "Data from the Micronucleus Assay (B1) were not entered due to extremely low frequency of PCEs even in the lowest dose. A protocol addendum was generated with the consent of the Sponsor to perform another Range-Finding Assay with lower dose levels." Page 15 says "the slides were not scored because of extremely low frequency of the PCEs even in the lowest dose level." Thus, doses in this experiment caused severe myelosuppression.

Test B2

Page 18 of the study report says "Micronucleus Assay B2 was aborted because the dosing solutions from the assay were not able to be analyzed due to leakage during the shipment."

Test B3

The following tables from the study report shows the results of test B3 in male and female mice. Again the IC_{50} for males for the doses in this study was 3 mg/kg (estimated in GraphPad, $r^2 = 0.38$, not estimated for females). The highest dose in this experiment was 3 mg/kg, equal to the IC_{50}.

SUMMARY OF MICRONUCLEUS ASSAY RESULTS
Mean Percent PCE and Incidence of
MPCEs in Bone Marrow of Male Mice
Intraperitoneally Injected with PDX (PRALATREXATE)

Study No.: 0902-1521 B3 **Vehicle: Phosphate Buffered Saline (10 mL/kg)**

Time (hours)	Dose (mg/kg)	Cell Counts		PERCENT PCE	Change in %PCE***	MPCE for 2000 PCE
		PCE	NCE			
24	Vehicle	133	67	66.7	-	0.6
24	0.5	111	89	55.4	-16.9 %	0.2
24	1.0	102	98	51.1	-23.4 %	0.6
24	2.0	128	72	63.8	-4.3 %	1.4
24	3.0	75	125	37.7	-43.5 %	1.6
24	CP*	100	100	50.0	-25.0 %	52.6 **

NOTE: Five animals were used per group.
* CP was used as positive control and was dosed at 80 mg/kg by oral gavage.
** These results are considered statistically significant because the p-value is less than or equal to 0.025.
*** Change of Percent PCE in comparison with concurrent vehicle, calculated by the following formula:

$$\frac{\text{Percent PCE for Test Dose} - \text{Percent PCE for vehicle}}{\text{Percent PCE for vehicle}} \times 100$$

SUMMARY OF MICRONUCLEUS ASSAY RESULTS
Mean Percent PCE and Incidence of
MPCEs in Bone Marrow of Female Mice
Intraperitoneally Injected with PDX (PRALATREXATE)

Study No.: 0902-1521 B3 **Vehicle: Phosphate Buffered Saline (10 mL/kg)**

Time (hours)	Dose (mg/kg)	Cell Counts		PERCENT PCE	Change in %PCE***	MPCE for 2000 PCE
		PCE	NCE			
24	Vehicle	134	66	66.8	-	0.8
24	0.5	123	77	61.6	-7.8 %	0.4
24	1.0	109	91	54.6	-18.3 %	1.0
24	2.0	93	107	46.7	-30.1 %	0.6
24	3.0	86	114	43.0	-35.6 %	0.6
24	CP*	99	101	49.5	-25.9 %	46.8 **

NOTE: Five animals were used per group.
* CP was used as positive control and was dosed at 80 mg/kg by oral gavage.
** These results are considered statistically significant because the p-value is less than or equal to 0.025.
*** Change of Percent PCE in comparison with concurrent vehicle, calculated by the following formula:

$$\frac{\text{Percent PCE for Test Dose} - \text{Percent PCE for vehicle}}{\text{Percent PCE for vehicle}} \times 100$$

2.6.6.5 Carcinogenicity

The sponsor did not study the carcinogenic potential of pralatrexate.

2.6.6.6 Reproductive Toxicology

1) Pralatrexate (PDX): Intravenous-Dose Range Study for Effects on Embryo-Fetal Development in Sprague Dawley Rats

Major findings

In this dose range finding study in rats, the high dose of 2 mg/kg/day during gestation (12 mg/m^2/day or 40% of the single clinical dose on a mg/m^2 basis) resulted in two pregnant rats becoming moribund by day 20 of gestation. There was a dose dependant decrease in body weight gain in all dosed groups that was significant in animals dosed with 0.1, 0.3 or 2 mg/kg (about 36% less than controls in high dose animals). Three high dose animals showed signs of gross damage in the GI tract. Other gross findings in this group included adrenal gland enlargement, discolored liver, mandibular and mesenteric lymph nodes, lung or kidney, rough spleen, and a discolored thymus reduced in size.

Only one of eight animals in the 0.1 mg/kg dose group carried successfully to necropsy, all other animals in this group and all in the two higher dose groups totally resorbed their litters. Pralatrexate is a potent abortifacient.

Study no: Sponsor – PDX-T-07048-R; Laboratory – 1128-06470
File name: pdxt07048r—nonclinical-data.pdf
Laboratory: (b) (4)
Study Date: February 2007
GLP: Yes
Audited: Yes
Drug: Pralatrexate Injection, Lot 132I0606, Purity 98.14 %

Methods:
Species: Sprague Dawley Rats, 203 to 247 grams, age at first dose 8 to 10 weeks
Doses: The following table from the study report shows the doses, N = 8/group

Text Table 4: Study Design

Group	Treatment	Dose (mg/kg/day)	Dose Concentration (mg/mL)	Dose Volume (mL/kg)	Animal Groups and ID Numbers	
					Main	Toxicokinetic
1	PBS	0.0	0.0	1.28	4184-4191	None
2	PDX	0.03	0.02	1.28	4192-4199	4200-4208
3	PDX	0.1	0.08	1.28	4209-4216	4217-4225
4	PDX	0.3	0.23	1.28	4226-4233	4234-4242
5	PDX	2.0	1.56	1.28	4243-4250	4251-4259

Doses: 0, 0.18, 0.6, 1.8 and 12 mg/m^2
Treatment Course: Once daily from gestation day (GD) 7 to 20
Toxicokinetic satellite group GD 7 to 13
Route: IV tail vein bolus

Toxicokinetics: Predose, 0.5, 1, 4, 12 and 24 hours
nine rats/dose group, 2 samples per rat

Results

Toxicokinetics: The following table demonstrates that dosing was associated with systemic exposure in all dose groups.

Group	Dose (mg/kg/day)	Dose mg/m^2/day	C_{max} (nM)	T_{max} (h)	T_{last} (h)	AUC_{last} (nM*hr)
PDX 10-A						
2	0.03	0.18	8.6	0	1	6.5
3	0.1	0.6	29.9	0.5	1	16.8
4	0.3	1.8	45.2	0.5	1	28.7
5	2	12	219.9	0.5	1	137.2
PDX 10-B						
2	0.03	0.18	7.8	0.5	1	6.0
3	0.1	0.6	26.2	0.5	1	14.7
4	0.3	1.8	48.6	0.5	4	69.3
5	2	12	224.1	0.5	4	282.7
PDX_{total}						
2	0.03	0.18	13.9	0.5	1	12.4
3	0.1	0.6	56.1	0.5	1	32.9
4	0.3	1.8	93.8	0.5	4	135.5
5	2	12	444.0	0.5	4	580.1

PDX_{total} is the sum of PDX 10-A and PDX 10-B

The following graph shows that AUC increases with increases with increasing dose but the slope of the curve of the dose normalized AUC with verses dose is less than 1 (see toxicokinetics section above). C_{max} also increased linearly with dose. T_{last} was never greater than four hours; concentrations fell below limit of detection rapidly, limiting the number of data points and confounding the determination of pharmacokinetic parameters, particularly terminal half-life.

---

2) Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in Sprague Dawley Rats

Major findings

A dose of 0.06 mg/kg/day (0.36 mg/m^2/day or about 1.2% of the single clinical dose on a mg/m^2 basis) caused a decrease in weight gain relative to controls of about 8%; the medium dose, 0.3 mg/kg, caused a decrease in weight gain of about 6%. There was no other apparent maternal toxicity.

Treatment with PDX caused a dose dependant decrease in fetal viability that reached statistical significance in the HD group. This decrease manifested as an increase in late, early and total resorptions. There was also a dose dependant increase in percent post implantation loss and total post implantation loss that was statistically significant in the high dose group. The mean number of live fetuses and the mean number of live males and females decreased with increasing

dose. Fetal weight parameters decreased with increasing dose; these decreases were significant in the HD group.

While there appeared to be more total fetal anomalies in dosed animals there were frequently similar anomalies in controls. The incidence of no single anomaly reach statistical significance. The lack of obvious teratological effects may result from the increased mortality in dosed animals.

Study no: Sponsor – PDX-T-07050-R; Laboratory – 1128-06471
File name: pdxt07050r—nonclinical-data.pdf
Laboratory: (b) (4)
Study Date: March 2007
GLP: Yes
Audited: Yes
Drug: Pralatrexate Injection, Lot 132I0606, Purity 98.14 %

Methods:
Species: Sprague Dawley Rats, 203 to 243 grams, age at first dose 8 to 10 weeks
Doses: The following table from the study report shows the doses and N

Text Table 4: Study Design

Group	Treatment	Dose (mg/kg/day)	Dose Concentration (mg/mL)	Dose Volume (mL/kg)	Females
1	PBS	0.0	0.0	1	25
2	PDX	0.01	0.01	1	25
3	PDX	0.03	0.03	1	25
4	PDX	0.06	0.06	1	25

Doses: 0.06, 0.18 and 0.36 mg/m^2
Treatment Course: once daily from gestation day (GD) 7 to 20
Route: IV tail vein bolus
Formulation: PBS
Cage side: Twice daily
Clinical signs: GD 7, 11, 14, 17, 20, and 22
Body weights: GD 7, 11, 14, 17, 20, and 22
Food consumption: GD 7, 11, 14, 17, 20, and 22
Necropsy: GD 22
Uterine exam: At Necropsy
Fetal Evaluation: At Necropsy

Results:

Mortality: All does survived to scheduled necropsy
Clinical Observations: No toxicologically significant changes
Body Weight: The following graph shows that rats in the mid and high dose groups gained less weights than controls over the course of the study. The differences between the control values and those in the HD group were statistically significant on days 20 and 22 (6% and 8% decrease respectively).
Food Consumption: Decreased in the HD group as much as 10%, statistically significant on days 20 and 22.
Gross Pathology: There were no toxicologically significant findings among the does

Pregnancy: All the animals were pregnant at the time of necropsy
All animals had live fetuses at the time of necropsy
None of the fetuses were aborted in any dose group

Uterine findings: The following table shows the uterine weight (with cervix and ovaries) and the adjusted body weight for all groups. Uterine weight decreased with increasing dose and was significant in the HD group.

Group		Uterine Weights (g)	% Change	Total Body Weight Change Adjustedfor Gravid Uterine Weights (g)	% Change
Control	Mean	92.49	0.0%	37.82	0.0%
	S.D.	10.64		9.83	
	N	25		25	
LD	Mean	89.32	-3.4%	34.55	-8.6%
	S.D.	12.04		14.75	
	N	25		25	
MD	Mean	84.3	-8.9%	38.07	0.7%
	S.D.	20.95		15.1	
	N	25		25	
HD	Mean	**70.02**	-24.3%	34.42	-9.0%
	S.D.	20.78		16.1	
	N	25		25	

The following table from the study report shows the summary fetal data:

Allos Therapeutics, Inc. (b) (4) **Study No. 1128-06471**

Table 9
Summary of Uterine Data
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in Sprague Dawley Rats

Group		Number of Corpora Lutea	Number of Imp.	% pre-Imp. Loss	--- Resorptions--- Late	Early	Total	Dead Fetus	Aborted Fetus	Total Post Imp. Loss	Post Imp. Loss	Total Live Fetuses	% of Viable Imp.
1	TOTAL	320	298		0	3	3	0	0	3		295	
	MEAN	12.8	11.9	6.4(6.9)	0.0	0.1	0.1	0.0	0.0	0.1	1.1(1.0)	11.8	98.9(99.0)
	SD	1.96	1.68		0.00	0.33	0.33	0.00	0.00	0.33		1.78	
2	TOTAL	325	299		0	15	15	0	0	15		284	
	MEAN	13.0	12.0	7.7(8.0)	0.0	0.6	0.6	0.0	0.0	0.6	5.3(5.0)	11.4	95.0(95.0)
	SD	1.89	1.74		0.00	1.00	1.00	0.00	0.00	1.00		1.89	
3	TOTAL	300	277		0	12	12	0	0	12		265	
	MEAN	12.0	11.1	7.7(7.7)	0.0	0.5	0.5	0.0	0.0	0.5	5.5(4.3)	10.6	94.5(95.7)
	SD	2.42	2.43		0.00	0.87	0.87	0.00	0.00	0.87		2.53	
4	TOTAL	325	296		7*	50*	57*	0	0	57*		239	
	MEAN	13.0	11.8	8.6(8.9)	0.3	2.0	2.3	0.0	0.0	2.3	10.4*(19.3)	9.6*	80.6*(80.7)
	SD	1.96	1.75		0.61	2.16	2.30	0.00	0.00	2.30		2.86	

Imp. - Implantation * - Significantly different from the control value, p<0.05 . - Not applicable
Note: Figures in parentheses denote group percentage values

Nominal Dose: Group 1 - 0 mg/kg/day Group 2 - 0.01 mg/kg/day Group 3 - 0.03 mg/kg/day Group 4 - 0.06 mg/kg/day

Formulas for calculations:

% Pre-Imp. Loss = $\frac{\text{total number of corpora lutea - total number of implantations}}{\text{total number of corpora lutea}}$ X 100

% Imp. Resorption = 100 - $\frac{\text{total number of implantations - total number of resorptions}}{\text{total number of implantations}}$ X 100

% Post Imp. Loss = 100 - $\frac{\text{total number of implantations - total post implantation loss}}{\text{total number of implantations}}$ X 100

% of Implants Live = $\frac{\text{total number of live fetuses}}{\text{total number of implantations}}$ X 100

Best Available Copy

Treatment with PDX caused a dose dependant decrease in fetal viability. This decrease manifested as a statistically significant increase in late, early and total resorptions in the high dose group. This increase was apparent in the other two dose groups but did not reach statistical significance. There was a dose dependant increase in percent post implantation loss and total post implantation loss that was statistically significant in the high dose group. Consequently the percentage of viable implants diminished with dose and was significant in the high dose group.

The following table from the study report shows that the mean number of live fetuses and the mean number of live males decreased with increasing dose. The difference was significant in the HD group. The mean number of live females also decreased but did not reach significance. The table also shows that fetal weight parameters decreased with increasing dose and these decreases were significant in the HD group.

Table 10
Summary of Fetal Weights
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in Sprague Dawley Rats

	Group:	1	2	3	4
Mean Number of live fetuses per litter	Mean	11.8	11.4	10.6	9.6*
	S.D.	1.78	1.89	2.83	2.86
	N	25	25	25	25
Mean Number of male fetuses per litter	Mean	6.4	6.2	5.9	4.8*
	S.D.	1.73	2.22	2.10	1.65
	N	25	25	25	25
Mean Number of female fetuses per litter	Mean	5.4	5.1	4.7	4.7
	S.D.	2.16	1.64	2.46	2.28
	N	25	25	25	25
Mean % male fetuses	Mean	54.48	54.28	58.15	51.64
	S.D.	14.74	14.77	19.38	13.08
	N	25	25	25	25
Mean litter weight (g)	Mean	67.780	64.998	60.727	48.534*
	S.D.	8.278	9.625	15.919	17.323
	N	25	25	25	25
Mean fetal weight per litter (g)	Mean	5.774	5.746	5.735	4.982*
	S.D.	0.328	0.320	0.453	0.629
	N	25	25	25	25
Mean male fetal weight per litter (g)	Mean	5.915	5.887	5.856	5.022*
	S.D.	0.338	0.317	0.484	0.753
	N	25	25	25	25
Mean female fetal weight per litter (g)	Mean	5.601	5.580	5.639	4.947*
	S.D.	0.316	0.364	0.352	0.594
	N	25	25	25	25

* - Significantly different from the control value, $p<0.05$

The following table from the study report shows the fetal anomalies found after dosing with PDX.

Table 11
Summary of Fetal Examinations
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in Sprague Dawley Rats

	Classification	Group: 1	2	3	4
Total number of fetuses examined		295	284	265	239
External (rodent) Defects:					
Number of Fetuses Examined		295	284	265	239
Number of Litters Examined		25	25	25	25
Hindlimb					
Paw, Right, Syndactyly	Malformation	.	.		1(0.4)
		.	.		1(4.0)
Head					
Eye, Left, Open eye	Malformation	.	.	1(0.4)	.
		.	.	1(4.0)	.
Forelimb					
Paw, Bilateral, Brachydactyly	Malformation	.	.		1(0.4)
		.	.		1(4.0)
Body general					
General, Edema	Variation	.	.		1(0.4)
		.	.		1(4.0)

. - Not applicable
Note: Upper line denotes number of affected fetuses. Lower line denotes number of affected litters. Figures in parentheses denote percentage incidence. Only abnormal findings are presented.

Best Available Copy

Table 11 (continued)
Summary of Fetal Examinations
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in Sprague Dawley Rats

	Classification	Group: 1	2	3	4
Fresh visceral body Defects:					
Number of Fetuses Examined		142	142	126	111
Number of Litters Examined		25	25	25	25
Abdomen					
Ureter, Left, Dilated ureter	Variation	1(0.7) 1(4.0)	1(0.7) 1(4.0)	. .	. .
Ureter, Right, Dilated ureter	Variation	1(0.7) 1(4.0)	. .	2(1.6) 2(8.0)	3(2.7) 2(8.0)
Ureter, Bilateral, Dilated ureter	Variation	. .	4(2.8) 1(4.0)	. .	. .
Fetus					
General, Situs inversus	Malformation	1(0.7) 1(4.0)	. .	. .	. .
Cardiovascular System					
Inominate artery, Short innominate	Variation	. .	2(1.4) 1(4.0)	. .	1(0.9) 1(4.0)

. - Not applicable
Note: Upper line denotes number of affected fetuses. Lower line denotes number of affected litters. Figures in parentheses denote percentage incidence. Only abnormal findings are presented.

Table 11 (continued)
Summary of Fetal Examinations
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in Sprague Dawley Rats

	Classification	Group: 1	2	3	4
Bouin's head Defects					
Number of Fetuses Examined		142	140	126	111
Number of Litters Examined		25	25	25	25
Head					
Brain, Hydrocephaly	Malformation	. .	. .	. .	2(1.8) 1(4.0)
Brain, Dilated cerebral ventricle	Variation	. .	. .	. .	2(1.8) 1(4.0)
Skeletal (rodent) Defects					
Number of Fetuses Examined		153	144	139	128
Number of Litters Examined		25	25	25	25
Ribs					
Rib, Bilateral, Bent	Variation	1(0.7) 1(4.0)	. .	. .	. .
Rib, Bilateral, Knobby rib	Variation	. .	. .	1(0.7) 1(4.0)	. .
Rib, Bilateral, Short supernumerary rib	Variation	3(2.0) 3(12.0)	2(1.4) 2(8.0)	4(2.9) 3(12.0)	. .
Rib, Bilateral, Wavy rib	Variation	1(0.7) 1(4.0)	. .	1(0.7) 1(4.0)	1(0.8) 1(4.0)
Rib, Left, Short supernumerary rib	Variation	3(2.0) 3(12.0)	3(2.1) 3(12.0)	3(2.2) 3(12.0)	3(2.3) 3(12.0)

. - Not applicable
Note: Upper line denotes number of affected fetuses. Lower line denotes number of affected litters. Figures in parentheses denote percentage incidence. Only abnormal findings are presented.

Best Available Copy

Table 11 (continued)
Summary of Fetal Examinations
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in Sprague Dawley Rats

	Classification	Group: 1	2	3	4
Skeletal (rodent) Defects					
Number of Fetuses Examined		153	144	139	128
Number of Litters Examined		25	25	25	25
Ribs					
Rib, Right, Full supernumerary rib	Variation	.	.	1(0.7)	.
		.	.	1(4.0)	.
Rib, Right, Short supernumerary rib	Variation	4(2.6)	1(0.7)	2(1.4)	.
		4(16.0)	1(4.0)	2(8.0)	.
Sternebrae					
General, Bipartite ossification	Variation	.	1(0.7)	1(0.7)	2(1.6)
		.	1(4.0)	1(4.0)	1(4.0)
General, Extra site of ossification	Variation	.	.	1(0.7)	.
		.	.	1(4.0)	.
General, Incomplete ossification	Variation	.	.	.	2(1.6)
		.	.	.	2(8.0)
General, Unossified	Variation	.	2(1.4)	3(2.2)	3(2.3)
		.	2(8.0)	3(12.0)	3(12.0)
Vertebral column					
Centrum, Caudal, Unossified	Variation	.	.	1(0.7)	1(0.8)
		.	.	1(4.0)	1(4.0)

. = Not applicable
Note: Upper line denotes number of affected fetuses. Lower line denotes number of affected litters. Figures in parentheses denote percentage incidence. Only abnormal findings are presented.

Table 11 (continued)
Summary of Fetal Examinations
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in Sprague Dawley Rats

	Classification	Group: 1	2	3	4
Skeletal (rodent) Defects					
Number of Fetuses Examined		153	144	139	128
Number of Litters Examined		25	25	25	25
Vertebral column					
Centrum, Sacral, Fused	Malformation	.	.	.	1(0.8)
		.	.	.	1(4.0)
Centrum, Thoracic, Bipartite ossification	Variation	1(0.7)	1(0.7)	.	2(1.6)
		1(4.0)	1(4.0)	.	2(8.0)
Centrum, Thoracic, Bipartite ossification	Variation	.	.	1(0.7)	.
		.	.	1(4.0)	.
Centrum, Thoracic, Hemicentric	Variation	.	.	1(0.7)	.
		.	.	1(4.0)	.
Centrum, Thoracic, Unossified	Variation	.	.	1(0.7)	.
		.	.	1(4.0)	.
Vertebra, Caudal, Unossified	Malformation	.	.	.	2(1.6)
		.	.	.	1(4.0)
Skull					
Hyoid, Unossified	Variation	.	1(0.7)	1(0.7)	1(0.8)
		.	1(4.0)	1(4.0)	1(4.0)

. = Not applicable
Note: Upper line denotes number of affected fetuses. Lower line denotes number of affected litters. Figures in parentheses denote percentage incidence. Only abnormal findings are presented

Best Available Copy

Table 11 (continued)
Summary of Fetal Examinations
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in Sprague Dawley Rats

	Classification	Group: 1	2	3	4
Skeletal (rodent) Defects					
Number of Fetuses Examined		153	144	139	128
Number of Litters Examined		25	25	25	25
Skull					
Supraoccipital, Incomplete ossification	Variation	.	1(0.7)	4(2.9)	.
		.	1(4.0)	2(8.0)	.
Squamosal, Right, Incomplete ossification	Variation	.	.	1(0.7)	.
		.	.	1(4.0)	.
Interparietal, Incomplete ossification	Variation	.	.	1(0.7)	.
		.	.	1(4.0)	.
Parietal, Right, Incomplete ossification	Variation	2(1.3)	.	.	.
		2(8.0)	.	.	.
Parietal, Bilateral, Incomplete ossification	Variation	.	.	1(0.7)	.
		.	.	1(4.0)	.
Frontal, Right, Incomplete ossification	Variation	.	.	1(0.7)	.
		.	.	1(4.0)	.
Frontal, Bilateral, Incomplete ossification	Variation	.	.	3(2.2)	.
		.	.	2(8.0)	.
Maxilla, Bilateral, Incomplete ossification	Variation	.	.	1(0.7)	.
		.	.	1(4.0)	.

. - Not applicable
Note: Upper line denotes number of affected fetuses. Lower line denotes number of affected litters. Figures in parentheses denote percentage incidence. Only abnormal findings are presented

Best Available Copy

3) Pralatrexate (PDX): Intravenous-Dose Range Study for Effects on Embryo-Fetal Development in New Zealand White Rabbits

Major findings

In this range finding study in rabbits, one control, one low mid dose (12 mg/m^2), two high mid dose (36 mg/m^2) and one high dose (120 mg/m^2) does aborted between weeks 22 and 26. The high dose group in this study is about 4 times the single clinical dose on a mg/m^2 basis. Three of eight LMD and all HMD and HD animals had red vaginal discharge from days 18 to 24. Dosing was not associated with a decrease in body weight gain but food consumption was significantly greater in LMD, HMD and HD animals ($p < 0.05$ as much as 35% of total consumption in HD animals). There were no significant gross findings. All animals became pregnant. One control, three LMD and all HMD and HD animals totally resorbed their litters.

Study no:	Sponsor – PDX-T-07047-B; Laboratory – 1128-06473
File name:	pdxt07047b—nonclinical-data.pdf
Laboratory:	(b) (4)
Study Date:	January 2007
GLP:	Yes
Audited:	Yes
Drug:	Pralatrexate Injection, Lot 132I0606, Purity 98.14 %
Methods:	
Species:	Female New Zealand White Rabbits, 2989 to 3704 g, age at first dose 6 to 7 months
Doses:	The following table from the study report shows the doses and N=8

Text Table 4: Study Design

Group	Treatment	Dose (mg/kg/day)	Dose Concentration (mg/mL)	Dose Volume (mL/kg)	Animal Groups and ID Numbers	
					Main	Toxicokinetic
1	PBS	0.0	0.0	1.0	3974-3981	None
2	PDX	0.03	0.03	1.0	3982-3989	3990-3992
3	PDX	1.0	1.0	1.0	3993-4000	4001-4003
4	PDX	3.0	3.0	1.0	4004-4011	4012-4014
5	PDX	10.0	10.0	1.0	4015-4022	4023-4025

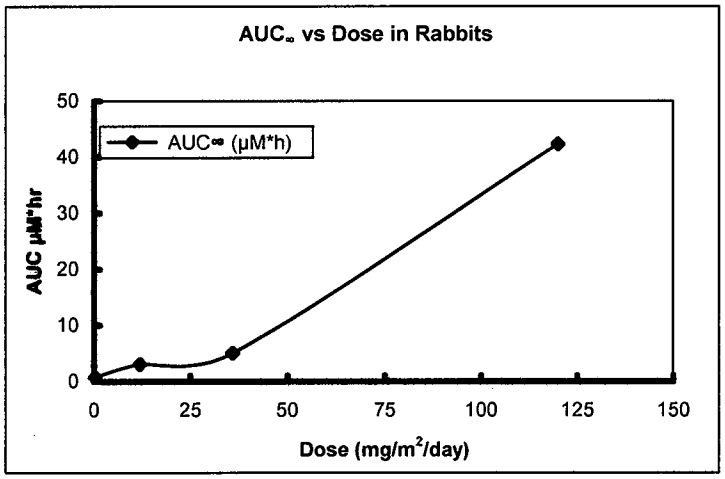
Doses: 0, 0.24, 12, 36 or 120 mg/m^2
Treatment Course: Once daily from gestation day (GD) 8 to 21
Route: IV bolus, ear vein
Formulation: PBS
Cage side: Twice daily
Clinical signs: GD 8, 11, 14, 17, 21, 24, 27, 30
Body weights: Daily
Food consumption: Daily
Toxicokinetics: GD 14 and 15, predose, 0.5, 1, 4, 12, and 24 hours post dose
Necropsy: After apparent abortion and on GD 30
Uterine exam: At necropsy
Fetal Evaluation: At necropsy

Results:

Toxicokinetics

Group	Dose (mg/kg/day)	Dose (mg/m^2/day	C_{max} (µM)	T_{max} (h)	T_{last} (h)	$AUCl_{ast}$ (µM*h)	AUC_{∞} (µM*h)	$t_{1/2}$ (h)
PDX 10-A								
2	0.03	0.36	0.55	0.5	4	0.46	0.46	0.44
3	1	12	1.19	0.5	4	1.59	1.59	0.42
4	3	36	2.43	0.5	4	2.30	2.32	0.43
5	10	120	17.13	0.5	4	18.09	18.09	0.35
PDX 10-B								
2	0.03	0.36	0.44	0.5	4	0.35	0.35	0.46
3	1	12	1.13	0.5	4	1.47	1.47	0.41
4	3	36	2.93	0.5	4	2.74	2.74	0.42
5	10	120	21.78	0.5	4	24.08	24.29	0.34
PDX_{total}								
2	0.03	0.36	0.99	0.5	4	0.81	0.81	0.45
3	1	12	2.32	0.5	4	3.06	3.06	0.41
4	3	36	5.36	0.5	4	5.05	5.05	0.42
5	10	120	38.95	0.5	4	42.31	42.31	0.34

A graph of AUC verses dose shows that AUC did not increase linearly in this experiment, but reached a plateau and at 36 mg/m^2 then increased sharply to 120 mg/m^2. This suggests the possibility of the saturation of some metabolic mechanism. The C_{max} and AUC values for of PDX 10A and PDX 10-B were comparable.



4) Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in New Zealand White Rabbits

Major findings

A high dose of 1 mg/kg/day (12 mg/m^2/day or about 40% of the single clinical dose) caused three rabbits to completely abort their litters. This dose caused red vaginal discharge between days 18 and 25 in seven of 25 animals. Mid and high dose (0.1 mg/kg) animals gained less weight than controls but this difference was small (-0.1% and -2.5% respectively). The high dose caused no other systemic maternal toxicity.

All drug treated animals became pregnant. In eight high dose does the litters were completely resorbed at necropsy compared to one each in all other groups. There was a statistically significant decrease in uterine weight (41% $p < 0.05$). Early and total resorptions were significantly greater in the high dose animals than in other groups. Post implantation loss was increased and the total number of live fetuses was decreased in this group. There was no increase in morphological defects among fetuses treated with pralatrexate compared to controls. Nevertheless, the number of fetuses in the high dose group was only 50 compared to 154 controls.

Study no:	Sponsor – PDX-T-07051-B; Laboratory – 1128-06473
File name:	pdxt07051b—nonclinical-data.pdf
Laboratory:	(b) (4)
Study Date:	March 2007
GLP:	Yes
Audited:	Yes
Drug:	Pralatrexate Injection, Lot 132I0606, Purity 98.14 %
Methods:	
Species:	Female New Zealand White Rabbits, 3009 to 3867 g, age at first dose 6 to 7 months
Doses:	The following table from the study report shows the doses and N

Text Table 4: Study Design

Group	Treatment	Dose (mg/kg/day)	Dose Concentration (mg/mL)	Dose Volume (mL/kg)	Animal Numbers
1	PBS	0.0	0.0	1.0	4026-4045
2	PDX	0.03	0.03	1.0	4046-4065
3	PDX	0.1	0.1	1.0	4066-4085
4	PDX	1.0	1.0	1.0	4086-4105

Doses: 0, 0.36, 1.2 and 12 mg/m^2
Treatment Course: Once daily from gestation day (GD) 8 to 21
Route: IV bolus, ear vein
Formulation: PBS
Cage side: Twice daily
Clinical signs: GD 8, 11, 14, 17, 20, 23, 26, 29 and prior to termination
Body weights: GD 8, 11, 14, 17, 20, 23, 26, 29 and prior to termination
Food consumption: Daily
Necropsy: After apparent abortion and on GD 30
Uterine exam: At necropsy
Fetal Evaluation: At necropsy

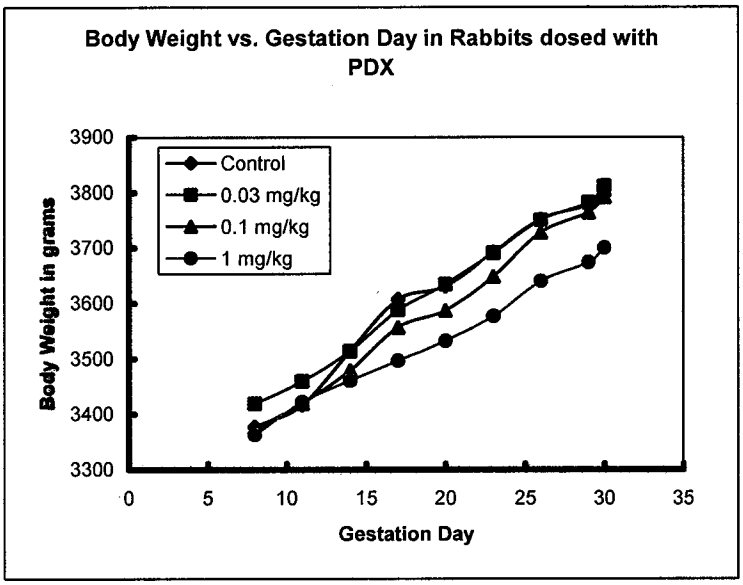
Protocol deviations: Animals 4057 (2F) and 4077 (3F) were given water bottles and a supplemental diet of kale and alfalfa for 3 to 7 days as a result of veterinary evaluations.

Results:

Mortality: Three HD does were necropsied between GD 21 and 24 because of apparent abortion of their litters. All other animals survived to scheduled necropsy

Clinical Observations: One MD and 7 HD does had red vaginal discharge between days 18 and 25

Body Weight: The following graph demonstrates a dose dependant decrease in weight gain in mid dose and high dose rabbits. While quite apparent this decrease did not reach statistical significance because of relatively large variability. The decrease was -0.1% in the mid dose animals and -2.5% in the high dose animals.



Food Consumption: Food consumption in mid dose and high dose animals increased (>35% in high dose animals on day 29) after the cession of dosing probably in compensation for the diminished weight gain

Gross Pathology: No maternal gross pathology

Pregnancy: The following table from the study report shows that all but one control doe became pregnant. Three high dose does aborted, 8 high-dose does had totally resorbed litters at necropsy compared to one each in all other groups.

Table 7
Summary of Pregnancy Data
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in New Zealand White Rabbits

Group	1	2	3	4
Number of animals in group	20	20	20	20
Not pregnant (%)	1(5.0)	0(0.0)	0(0.0)	0(0.0)
Died/Killed	0	0	0	0
Survived to scheduled kill	1	0	0	0
Pregnant (%)	19(95.0)	20(100.0)	20(100.0)	20(100.0)
Died/Killed/Aborted	0	0	0	3
with total resorption	1	1	1	8
with live fetuses at scheduled kill	18	19	19	12

Uterine findings: The following table from the study report shows a statistically significant decrease in uterine weight (41% $p < 0.05$) and body weight changes adjusted for gravid uterine weight in high dose animals. Uterine weights include the cervix and ovaries. Calculations include only animals that were pregnant at scheduled necropsy.

Table 8
Summary of Body Weight Change Adjusted for Gravid Uterine Weight
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in New Zealand White Rabbits

Group Sex		Uterine[a] Weights (g)	Body Weight Changes Adjusted for Gravid Uterine Weights (g)
1f	Mean	498.40	-83.90
	S.D.	88.47	117.33
	N	18	18
2f	Mean	520.38	-127.38
	S.D.	77.91	127.92
	N	19	19
3f	Mean	505.57	-76.09
	S.D.	143.85	126.57
	N	19	19
4f	Mean	292.23*	49.78*
	S.D.	157.46	124.89
	N	12	12

The following table from the study report shows that early and total resorptions were significantly greater in the high dose animals than in other groups. Post implantation loss was increased and the total number of live fetuses was decreased in this group.

Table 9
Summary of Uterine Data
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in New Zealand White Rabbits

Group		Number of Corpora Lutea	Number of Imp.	% pre-Imp. Loss	--- Resorptions --- Late	Early	Total	Dead Fetus	Aborted Fetus	Total Post Imp. Loss	% Post Imp. Loss	Total Live Fetuses	% of Viable Imp.
1	TOTAL	179	163		1	8	9	0	0	9		154	
	MEAN	9.9	9.1	7.8(8.9)	0.1	0.4	0.5	0.0	0.0	0.5	5.2(5.5)	8.6	94.8(94.5)
	SD	2.13	1.76		0.24	0.62	0.71	0.00	0.00	0.71		1.72	
2	TOTAL	191	181		1	3	4	0	0	4		177	
	MEAN	10.1	9.5	4.9(5.2)	0.1	0.2	0.2	0.0	0.0	0.2	2.2(2.2)	9.3	97.8(97.8)
	SD	1.78	1.54		0.23	0.37	0.42	0.00	0.00	0.42		1.60	
3	TOTAL	192	171		0	2	2	0	0	2		169	
	MEAN	10.1	9.0	12.1(10.9)	0.0	0.1	0.1	0.0	0.0	0.1	3.5(1.2)	8.9	96.5(98.8)
	SD	2.73	3.09		0.00	0.32	0.32	0.00	0.00	0.32		3.31	
4	TOTAL	125	106		0	56	56	0	0	56		50	
	MEAN	10.4	8.8	15.0(15.2)	0.0	4.7*	4.7*	0.0	0.0	4.7*	50.7*(52.8)	4.2*	49.3*(47.2)
	SD	1.98	2.17		0.00	3.20	3.20	0.00	0.00	3.20		2.66	

The following table from the study report reiterates the decrease in live male and female fetuses in the HD group and shows a decrease in mean litter weight with no effect on mean fetal weight.

Table 10
Summary of Fetal Weights
Pralatrexate (PDX): Intravenous Study for Effects on Embryo-Fetal Development in New Zealand White Rabbits

	Group	1	2	3	4
Mean Number of live fetuses per litter	Mean	8.6	9.3	8.9	4.2*
	S.D.	1.72	1.60	3.31	2.66
	N	18	19	19	12
Mean Number of male fetuses per litter	Mean	4.4	3.7	4.4	2.3*
	S.D.	1.46	1.77	1.71	2.15
	N	18	19	19	12
Mean Number of female fetuses per litter	Mean	4.1	5.6	4.5	1.8*
	S.D.	1.37	1.95	2.82	1.34
	N	18	19	19	12
Mean % male fetuses	Mean	51.98	39.77	54.91	54.46
	S.D.	13.48	18.87	22.34	29.80
	N	18	19	19	12
Mean litter weight (g)	Mean	350.384	360.422	359.440	183.356*
	S.D.	62.375	51.752	115.483	118.040
	N	18	19	19	12
Mean fetal weight per litter (g)	Mean	41.292	39.012	41.866	44.173
	S.D.	3.851	3.452	4.947	3.525
	N	18	19	19	12
Mean male fetal weight per litter (g)	Mean	41.593	39.642	42.461	46.456
	S.D.	3.825	5.054	4.979	4.316
	N	18	19	19	12
Mean female fetal weight per litter (g)	Mean	40.895	38.782	40.075	42.258
	S.D.	4.181	3.147	3.908	4.837
	N	18	19	19	12

Fetal Morphology:

While the incidence of malformations appeared somewhat higher subjectively in animals dosed with pralatrexate no incidence of morphologically malady was significantly greater than that observed in controls and there was no clear dose effect (data not shown). Nevertheless, the number of fetuses in the high dose group was only 50 compared to 154 controls.

2.6.6.7 Local tolerance

1) Perivascular and Intradermal Injection Effects of PDX in Sprague Dawley

Major findings

Intradermal injection of 20 mg/kg (120 mg/m^2) caused minimal light pink swelling in three of five male rats and 4 of 5 female rats at the injection site by day 8 after the injection. The reaction was somewhat more severe in females. A single perivascular injection (saphenous vein) of 40 mg/kg (240 mg/m^2) caused no changes in male or female rats at the injection site.

Study no: Sponsor – PDX-T-04003-R; Laboratory – 1128-121
File name: pdxt08064d—nonclinical–data.pdf
Laboratory: (b) (4)
Study Date: May 2004
GLP: No
Audited: No
Drug: Pralatrexate, Lot # 34011103, purity 97.9%
Methods:

Species:	Sprague Dawley rats, 9 weeks old
Doses:	0, treatment group 1
	20 mg/kg (120 mg/m^2) intradermal route, treatment group 2
	40 mg/kg (240 mg/m^2), perivascular route, treatment group 3
Concentration	20 mg/mL
N	5 per sex per test group
Schedule	Single dose
Route	Intradermal or perivascular (saphenous vien)
Dose volume	1 mL/kg intradermal route, 2 mL/kg perivascular route
Vehicle	PBS
Cage side	At least twice a day
Clinical Obs.	SD 1 and termination
Body Weight	SD 1 and termination
Evaluation	Draize Evaluation of injection sites prior to the first dose and daily thereafter until termination (scale of severity 0 to 4)
Necropsy	SD 8
Histopathology	Injection site only

Results

Mortality	All animals survived to scheduled necropsy
Clinical Signs	None
Body Weight	Increased normally in controls and treated males
	Increased in female controls and decreased slightly in treated females
Draize Evaluation	The following tables from the study report summarize the results for this evaluation in males and females respectively

Table 4
Summary of Draize Evaluations
Perivascular and Intradermal Injection Effects of PDX in Sprague Dawley Rats

Group Sex	Clinical Sign	Severity	Day Numbers Relative to Start Date 1	2	3	4	5	6	7	8
1m	ANIMALS ALIVE		5	5	5	5	5	5	5	5
	Edema (treated site - 1)	none; no swelling	5	5	5	5	5	5	5	5
	Edema (treated site - 2)	none; no swelling	5	5	5	5	5	5	5	5
	Erythema (treated site - 1)	none; normal color	5	5	5	5	5	5	5	5
	Erythema (treated site - 2)	none; normal color	5	5	5	5	5	5	5	5
2m	ANIMALS ALIVE		5	5	5	5	5	5	5	5
	Edema (treated site - 1)	none; no swelling	5	5	5	4	4	4	4	5
		minimal; slight swelling	.	.	.	1	1	1	1	.
	Erythema (treated site - 1)	none; normal color	5	5	5	5	4	4	3	2
		minimal;light pink	.	.	.	.	1	1	2	3
3m	ANIMALS ALIVE		5	5	5	5	5	5	5	5
	Edema (treated site - 2)	none; no swelling	5	5	5	5	5	5	5	5
	Erythema (treated site - 2)	none; normal color	5	5	5	5	5	5	5	5

m - male
. - not applicable
treated site - 1: Intradermal site (interscapular)
treated site - 2: Perivascular site (saphenous vein)
Nominal Dose: Group 1 - 0 mg/kg Group 2 - 20 mg/kg Group 3 - 40 mg/kg

Table 4 (continued)
Summary of Draize Evaluations
Perivascular and Intradermal Injection Effects of PDX in Sprague Dawley Rats

Group Sex	Clinical Sign	Severity	Day Numbers Relative to Start Date 1	2	3	4	5	6	7	8
1f	ANIMALS ALIVE		5	5	5	5	5	5	5	5
	Edema (treated site - 1)	none; no swelling	5	5	5	5	5	5	5	5
	Edema (treated site - 2)	none; no swelling	5	5	5	5	5	5	5	5
	Erythema (treated site - 1)	none; normal color	5	5	5	5	5	5	5	5
	Erythema (treated site - 2)	none; normal color	5	5	5	5	5	5	5	5
2f	ANIMALS ALIVE		5	5	5	5	5	5	5	5
	Edema (treated site - 1)	none; no swelling	5	5	5	5	3	3	4	3
		minimal; slight swelling	.	.	.	.	2	2	1	2
	Erythema (treated site - 1)	none; normal color	5	5	5	1	1	1	1	2
		minimal;light pink	.	.	.	3	2	2	4	3
		mild; bright pink/pale red	.	.	.	1	2	2	.	.
3f	ANIMALS ALIVE		5	5	5	5	5	5	5	5
	Edema (treated site - 2)	none; no swelling	5	5	5	5	5	5	5	5
	Erythema (treated site - 2)	none; normal color	5	5	5	5	5	5	5	5

f - female
. - not applicable
treated site - 1: Intradermal site (interscapular)
treated site - 2: Perivascular site (saphenous vein)
Nominal Dose: Group 1 - 0 mg/kg Group 2 - 20 mg/kg Group 3 - 40 mg/kg

Histopathology No toxicologically significant lesions

2.6.6.8 Special toxicology studies

1) Pdx-T-01007-B: Summary Report of the Safe Passage of 10-Propargyl-10-Deazaaminopterin (PDX) and Probenecid In Rabbits

In this study, investigators treated three New Zealand white rabbits with 40 mg/m^2 (3.3 mg/kg) pralatrexate and 70 mg/m^2 (5.8 mg/kg) probenecid (IV ear vein) on days 1 and 14. They collected blood samples from each rabbit on day 1 before the first injection, day 15, and day 17 for liver function evaluation. The rabbits were weighed twice weekly during the study and observed for a period of one-month post treatment.

The animals maintained normal weight during the experiment and showed no clinical signs of toxicity. There was no significant change in the liver enzyme levels. The results of all determinations were within the normal range for rabbits according to the reviewing veterinarian.

Study no:	Sponsor – PDX-T-01007-b
File name:	pdxt001007b—nonclinical-data.pdf
Laboratory:	Memorial Sloan Kettering Cancer Center, New York, NY
Study Date:	June 2001
GLP:	No
Audited:	No
Drug:	Pralatrexate Injection, Lot not specified

2) (b) (4): A Six-Week Intravenous Toxicity and Toxicokinetic Study in Beagle Dogs with a Two-Week Recovery Period

Major Findings

Dogs dosed intravenously with doses 3, 10 or 30 mg/kg (60, 200 or 600 mg/m^2) weekly for six weeks all vomited or showed signs of nausea shortly after dosing. This toxicity was subjectively worse in males than females. The time of onset suggests that this is an acute neurotoxicity. Two high dose males showed signs of other neurotoxicities shortly after dosing including ataxia, pale gums, labored breathing, lethargic and unresponsiveness.

Kidney-to-body weight ratios were higher in HD males. Grossly, the kidneys of one dog were enlarged. Microscopically all four HD dogs had some degree of "subtle cortical tubular dilation and chronic inflammation." Three of four 30-mg/kg males had tubular mineralization present in the kidney cortex. This damage was less severe or absent in recovery animals suggesting that it was reversible.

This study qualifies (b) (4) at the concentrations greater than the proposed drug product specifications.

Study no:	Sponsor – PDX-T-08064-D; Laboratory – 1128-08172
File name:	pdxt08064d—nonclinical–data.pdf
Laboratory:	(b) (4)
Study Date:	June 2008
GLP:	Yes
Audited:	Yes
Drug:	(b) (4), Lot BPH-K-55(13), Purity % 98.1
Methods:	
Species:	Beagle Dog, males 8.5 – 11.1 kg, females 5.1 – 8.8 kg Age at first dose 7 to 8 months
Doses:	0, 3, 10 or 30 mg/kg (0, 60, 200 or 600 mg/m^2) nominal
Concentration	0, 3, 10 or 30 mg/mL nominal 2.7 to 2.7, 9.0 to 9.4, 27.2 to 27.8 mg/mL assay
N	Four per sex per dose group in the low and mid dose groups 6 per sex per dose group in control and high dose (two per sex for recovery)
Schedule	Weekly for six weeks
Route	IV
Vehicle	0.9% saline
Body Wt.	Prior to dosing on SD 1, 8, 15, 22, 29 and 36, on the day prior to necropsy
Clinical obs.	Weekly
Ophthalmoscopy	Prior to dosing and prior to necropsy
Hematology	Prior to initiation of dosing and prior to necropsy
Clinical Chem	Prior to initiation of dosing and prior to necropsy
Coagulation	Prior to initiation of dosing and prior to necropsy
Urinalysis	Prior to initiation of dosing and prior to necropsy
ECG	Prior to dosing and within 15 minutes of the completion of dosing on SD 15 and 36
Toxicokinetics	prior to dosing, at 5, 10 and 30 minutes post-dose, and at 2, 4, 12 and 24 hours following the end of infusion on SD 1 and 36.
Necropsy	SD 43 and 57
Histopathology	Adequate battery

Results

Mortality	All animals survived to scheduled necropsy
Clinical Observations	All doses caused emesis within minutes of administration, at the highest dose nausea and emesis persisted for up to four hours and was more severe in males than females One HD male showed signs of ataxia, reduced capillary refill time, pale gums, and labored breathing. With subsequent doses, the severity diminished. Another HD male was lethargic following the fifth and sixth doses; the animal did not respond to touch or voice.
Body Weight	No toxicologically significant effects
Food Consumption	No toxicologically significant effects
Ophthalmology	No toxicologically significant effects
ECG	No toxicologically significant effects
Clinical Chemistry	No toxicologically significant effects
Hematology	No toxicologically significant effects
Coagulation	No toxicologically significant effects
Urinalysis	No toxicologically significant effects
Organ Weight	Kidney-to-body weight ratios were higher in HD males. This correlated bilateral tubular dilation seen microscopically. Other changes were incidental.
Gross Pathology	Bilateral enlargement of the kidneys of one HD male, correlated with microscopic evidence of tubular dilation. All other gross findings were incidental.
Histopathology	All four HD males had microscopic subtle cortical tubular dilation and chronic inflammation, and three of four 30-mg/kg males had tubular mineralization present in the kidney cortex. This damage was less severe or absent in recovery animals suggesting that it was reversible.
Toxicokinetics	See Toxicokinetic section above

2.6.6.9 Discussion and Conclusions

See below in Overall Conclusions and Recommendations and above in the Toxicology Summary

2.6.6.10 Tables and Figures

See above in individual studies reviews

2.6.7 TOXICOLOGY TABULATED SUMMARY

Study number	Study Type	Species	Schedule	Dose mg/kg	Dose mg/m²	mortality	Toxicity
PDX-T-05008-D	Acute toxicity	Dog	Single dose	0	0		
				3	60	2/2	GI toxicity
				6	120	2/2	GI toxicity
				9	180	2/2	GI toxicity
PDX-T-05013-R	Repeat dose	Rat	WeeklyX3	0	0		
			4 week course	25	150		GI toxicity
	Range finding		2 courses	50	300	3/5 F	GI toxicity
				75	450	4/5 F	GI toxicity
PDX-T-07034-R	Repeat dose	Rat	WeeklyX6	0	0		
			7 week course	5	30		
	Main study		7 courses	10	60		
				25	150		Doses in this study were too low to effectively demonstrate the toxicity of pralatrexate
PDX-T-05015-D	Repeat dose	Dog	WeeklyX3	0	0		
			4 week course	0.3	6		
	Range finding		2 courses	1	20		Dehydration and weight loss
				3	60	1/2 M, 1/2 F	GI toxicity and weight loss
PDX-T-07035-D	Repeat dose		WeeklyX6	0	0		
			7 week course	0.1	2		
	Range finding		7 courses	0.3	6		
				1	20	1/2 M, 2/2 F	Dose lowered to 0.7 with folate supplimentation. Deaths due to gastrointestinal toxicities including diarrhea, emesis, elecrotlyte imbalance, anorexia)
PDX-T-07054-D	Repeat dose		WeeklyX6	0	0		
			7 week course	0.1	2		
	Main study		7 courses	0.3	6		
				0.7	14	3/12 F, 2/12 M	Following the deaths of these dogs, the remaining animals were given supplimentary folate and B12. There were no more deaths. Death was associated with GI toxicity. Other toxicities included decreased white cell counts and anemia and some signs of hepa
PDX-T-06021-I	Ames test	bacteria	Single incubation	100 to 5000 µg/plate			Negative but this is expected with this drug class
PDX-T-06022-H	Chromosome A	CHO cells	Single incubation	0.1.25 to 1.0 µg/mL			Negative but this test is relatively insensitive to antifolates
PDX-T-06023-M	Micronucleus	mouse	dailyX3	0.5 to 3 mg/kg			Negative but the doses scored were lower than Red Book recommendations
PDX-T-07050-R	Segment II	Rat	DailyX15	0	0		
				0.1	0.6		resorptions, post-implantation loss
				0.03	0.18		resorptions, post-implantation loss
				0.06	0.36		significant resorptions, post-implantation loss, decreased fetal weight, decreased live fetuses
PDX-T-07051-B	Segment II	Rabbit	DailyX15	0	0		
				0.1	1.2		
				0.03	0.36		pre-implantation loss
				0.06	0.72		significant resorptions, post-implantation loss, decreased live fetuses

OVERALL CONCLUSIONS AND RECOMMENDATIONS

Mechanism of Action

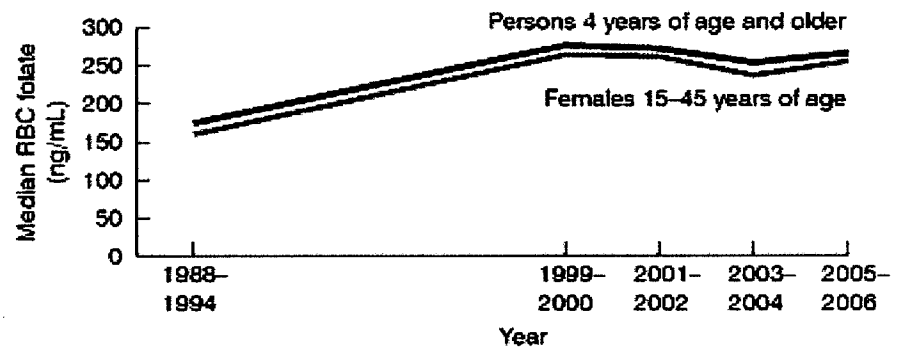
In 1993, J. I. DeGraw *et al.* (*J. Med. Chem.* 1993, **36,** 2228-2231) described the original synthesis of pralatrexate. Like aminopterin, an amino functional group replaces the hydroxy group on the pteridine ring. This single substitution is in itself sufficient to inhibit dihydrofolate reductase almost stoichiometrically though this inhibition is reversible (J.J. McGuire, *Current Pharmaceutical Design,* 2003, **9,** 2593-2613). This single change transforms the molecule from a vitamin to a potent cytotoxin. The amino group obviously interacts strongly with the THF binding site of DHFR. Pralatrexate differs from aminopterin in that the nitrogen in the 10 position is replaced by a methyl carbon which is further substituted with a propargyl group (propyl alkyne). The presence of a methyl group would by necessity prevent the methylation of the molecule to a methylated-tetrahydrofolate analogue because N^{10} is essential to form the methylene containing ring. These investigators further showed that pralatrexate inhibits dihydrofolate reductase with a k_i of 18 ± 4 pM; the k_i of methotrexate is 5.8 ± 1 pM so it is a more potent inhibitor. But surprisingly the k_i for transport influx of folate was 0.45 ± 0.1 μM while that of methotrexate is 4.2 ± 5 μM, almost ten fold higher. This advantage was reflected in the inhibition of the growth of mouse lymphoma cells *in vitro* where the IC_{50} for pralatrexate was 2 nM while that for methotrexate was 9.5 nM. Pralatrexate was also more effective than methotrexate at delaying tumor growth.

The presence of a terminal alkyne suggests the possibility that pralatrexate can react with biological electrophiles to form covalent bonds. If it did so with DHFR, it would be a suicide substrate, inhibition would not be non-competitive and DHFR function could only be restored by *de novo* synthesis. It could also react as a suicide substrate at the site of the folate transporter, RFC1 or folylpolyglutamate synthetase. None of these possibilities appear to have been investigated but this significant difference in structure could explain many of the differences between pralatrexate and other anti-folates.

In initial studies of the kinetics of methotrexate, inhibitory binding was thought to be non-competitive (double reciprocal plots did not meet at the ordinate) with very low inhibitory constants (see P.A. Frey and A. D. Hegeman, Enzymatic Reaction Mechanisms, Oxford University Press, 2007, page 271 and references therein). Subsequent experiments showed that the time course for inhibition for methotrexate and aminopterin inhibition was very slow; the half-time for dissociation (by dialysis) was six days in the absence of folate but only one day in its presence. Thus inhibition by methotrexate in the absence of folate was effectively non-competitive in the time range of most assays. The slow step in binding is due to a conformational change in DHFR (P. T. Ravi Rajagopalan *et al. PNAS.* October 15, 2002, **99**(21);13481–13486). Release is consequently slow. Thus, over multiple doses and in the absence of folate, DHFR becomes saturated and is released from inhibition only very slowly. Folate reserves pool as DHF and 5mTHF (*vide supra*) and for all purposes THF function can only be returned by *de novo* synthesis which itself is blocked by diminishing DNA function brought about by the inhibition of the purine synthesis. Thus the addition of folate can drastically alter the toxicity of antifolate drugs. In the same way it probably drastically diminishes their potential efficacy.

The mean concentration of folate in the blood of the U.S. population increased between 1988-1994 and 1999-2000. Small fluctuations in blood folate concentrations occurred between 1999-2006. The following graph shows this change.

Figure 1. Median RBC folate levels (ng/mL) of the U.S. population, 1988–2006



NOTES: Statistically significant difference between 1988–1994 and 1999–2000 for both groups. Statistically significant difference between 2001–2002 and 2003–2004 for both groups. Statistically significant difference between 2003–2004 and 2005–2006 for both groups. RBC is red blood cell.
SOURCE: CDC/NCHS, National Health and Nutrition Examination Surveys.

The median concentration of folate in red blood cells in the U.S. population four years old or older was 266 ng/mL (0.51 μM) in 2005-2006. The median serum folate concentration of the U.S. population 4 years of age and older was 12.2 ng/mL (0.028 μM) in 2005-2006 (National Center for Health Statistics brief - http://www.cdc.gov/nchs/data/databriefs/db06.htm Thus, the efficacy of antifolate drugs in the American population is possibly diminishing because of good folate nutrition.

Primary Pharmacodynamics

Pralatrexate inhibited the growth of a broad variety of human cancer cell types at relatively low concentrations (GI_{50} < 0.1 μM) after 48 hours exposure in the standard NCI *in vitro* assay. In other standard human tumor cell lines including HLaC, MDA-MB-231, MDA-MB-435, A549 and MV522 pralatrexate inhibited cell growth viability at mean concentrations between 12.8 and 65.1 nM (IC_{50} values). The compound inhibits other cell lines, MB-231, MDA-MB-435, NCI-H460, and SK-BR3 at mean concentrations between 6.7 and 54 nM

In an *in vitro* test for genotoxicity in mammalian cells, pralatrexate was cytotoxic to Chinese hamster ovary cells at concentrations above 10 μM. At lower concentrations the compound caused a dose dependant decrease in relative mitotic index but the cells maintained a normal morphology. This suggests that it is cytostatic at lower concentrations. This is consistent with its mechanism of action. The cells are prevented from entering S-phase by the paucity of thymidine. In the absence of metabolic activation, the IC_{50} was 1.4 μM for CHO cells in culture (3 hour exposure) based on RMI. The addition of S9 increased the IC_{50} to 4.3 μM. This increase in IC_{50} in the presence of S9 suggests that the microsomal enzymes within the S9 preparation are metabolizing pralatrexate to less toxic derivatives. With an 18 hour exposure, the compound did not prevent DNA synthesis as evidenced by the incorporation of BrdU within growing cells at concentrations less than or equal to 0.21 μM.

Safety Pharmacology

Pralatrexate caused changes in the action potential curve of dog Purkinje fibers only at concentrations many times higher than those attained clinically. Likewise it affected the hERG current in transfected CHO cells only at mM concentrations. Studies of cardiac toxicity in the

dog used doses only half as great as the clinical dose so the negative results of these studies are uninformative. Likewise studies of neurobehavioral changes in rats were only done at nontoxic doses.

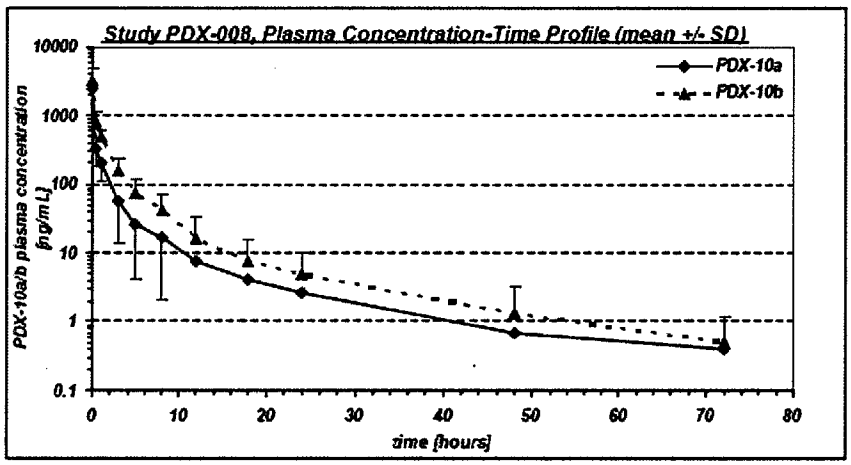
Toxicokinetics and Pharmacokinetics

The distribution and elimination of pralatrexate is complex. Plasma concentration verses time curves show three distinct phases, a rapid distribution phase, a slow partition phase and a very slow terminal elimination phase. This pattern is seen in male rats, male and female dogs and humans. The long terminal elimination phase is absent in female rats. The slow partition phase may represent enterohepatic recirculation but most likely represents redistribution into some compartment. None of the studies in this submission modeled the plasma elimination kinetics but M.P. Fanucchi *et al.* (*Cancer Res*, 1987, **47**:2334-2339) have shown that the elimination of 10-ethyl-10-deazaaminopterin, a compound very similar to pralatrexate, is triphasic by modeling. The terminal elimination half-life is 17 hours in humans, about 5 hours in dogs. The terminal elimination half-life in male rats is greater than 7 hours but in females it is less than an hour.

M.G. Lee *et al* (*Int J Clin Pharmacol Ther Toxicol.* 1984 Oct;**22**(10):530-7) have demonstrated that methotrexate exhibits unusual distribution kinetics in whole blood. They showed that plasma MTX concentrations generally dropped to a minimum at about 5 min then increased and fluctuated somewhat irregularly afterwards. They postulate that these unusual distribution phenomena might be due to the formation of Schiff bases between free primary amino groups of MTX and free fatty aldehyde groups on blood cell membrane. Thus whole blood may act as a reservoir for pralatrexate.

Folate can only enter the cell by means of the folate transporter. The mechanisms of this transporter are probably rate limiting in numerous processes in the liver and kidneys and may contribute to the complex elimination kinetics.

The following chart from the submission shows the plasma elimination curve for humans in study PDX-008. Curves in the animal studies were similar.



SD = standard deviation, PDX = pralatrexate, ng = nanogram, mL = milliliter

In most cases AUC and C_{max} increased linearly with dose and clearance was relatively invariant with dose. C_{max} at toxic or near toxic doses was in the 5 to 10 μM range. Clearance in rats was significantly higher (greater than 30 mL/min/kg without folate supplimentation) than it was in dogs and humans (about 10 mL/min/kg for dogs and 5.3 mL/min/kg for humans both with folate supplementation). Clearance in female rats was higher than that in male rats. These

clearance values are greater than renal or hepatic flow in rats but much less than renal or hepatic flow in humans and dogs. In humans about a third of a given dose is recovered in the urine in dogs about one fifth. None of the submitted studies adequately determined fecal elimination but it is likely that this is an important route. In all species volume of distribution at steady state was greater than total body weight consistent with the unusual distribution kinetics.

Little pralatrexate crosses the blood brain barrier (BBB). Plasma binding is about 67% and little of the drug crosses into RBCs.

Pooled human microsomes or S9 fraction do not appear to metabolize pralatrexate; neither do human hepatocytes *in vitro*. But in human liver microsomes, pralatrexate caused concentration dependant inhibition of the activity of cytochrome P450 2C19. Activity decreased to 71% at a concentration of 20 μM and was below detection at 50 μM or greater. Slight inhibition of the function of 2E1 and 2A6 (less than 30% decrease from the activity of control) was not concentration dependant. The compound did not cause dose dependant inhibition of cytochromes P450 1A2, 2A6, 2C9, 2D6 or 2E1. Pralatrexate did not induce an increase in the activity of cytochromes P450 1A2, 3A4 or 2C19 when incubated with fresh human hepatocytes. The results for the experiment with 2C19 are somewhat ambiguous since pralatrexate inhibits the activity of this enzyme in human microsomes.

The mass balance studies in this submission are uninformative because the radiolabel was on the terminal glutamate, the most labile portion of the molecule.

Toxicology

In the original IND submission, the initial toxicology studies done by MSKCC showed that 300 mg/kg (900 mg/m^2) of pralatrexate given weekly intravenously for three weeks did not cause any mortality in mice while a dose only one third greater killed 4 of 5 mice. On the same schedule, doses of 50 mg/kg (300 mg/m^2) were non-lethal, dose of 100 mg/kg (600 mg/m^2) were lethal to 1 of five male rats; while a dose only 50% greater (150 mg/kg) killed 60% of the rats. Again on the same schedule 8 mg/kg (160 mg/m^2) caused no mortality in 2/2 dogs but 12 mg/kg (240 mg/m^2) killed two of two dogs on days 2 and 14. The dose response curve for mortality is exceptionally steep being less than a factor of two between a reasonably safe dose and one that is rapidly fatal. The toxic dose response curves among the test species are also obviously shifted among species, the drug being more toxic to dogs than to rats at equivalent doses.

The serum concentrations of thymidine vary greatly among mammals. Using a unique microbiological assay, H Nottebrok and R.L. Then determined the serum concentrations across various species (*Biochemical Pharmacology*,1977, 26;2175-2179). The following table shows their results.

	Thymidine μg/mL	Thymidine μM	Ratio animal/man
Mouse	0.166	0.348	9.8
Rat	0.266	0.557	15.6
Rabbit	0.042	0.088	2.5
Dog	0.023	0.048	1.4
Monkey	0.056	0.117	3.3
Human	0.017	0.036	1

Other assays will show somewhat different values but the ratios between species are consistent. The table shows that the serum concentration of thymidine in rats and mice is at least 10 fold greater than that of humans and that in dogs is almost 1.5 times greater. Thus, rodents

have sufficient thymidine in normal circulation to significantly lessen the need for thymidine synthesis from uridine through the thymidine synthetase pathway. The dose response curve for toxicities due to anti-folates are shifted at least a factor of ten to the right in rats when compared to humans. Dogs have sufficient thymidine to shift the dose response curve such that this animal model is not as predictive as it usually is; nevertheless dogs are usually the only species that can predict a safe starting dose for anti-folates.

In the studies submitted to this NDA, three weekly doses over a four week course with two courses at a dose of 450 mg/m^2 caused 80% mortality female rats. Three hundred mg/m^2 was lethal to 60% of female rats. Thus, pralatrexate was somewhat more toxic in this study than it was in the earlier studies done by MSKCC. The most obvious reason for this is the increased number of courses, one verses two, and indeed much of the mortality occurred after the initiation of the second course in the Allos study. Other possible reasons could contribute to this difference in toxicity such as differences in the purity of the drug substance used by Allos and that used by MSKCC, differences in the strain of rat and the fact that the Allos study was GLP while the MSKCC study was not.

Doses of as high as 25 mg/kg (150 mg/m^2) given once weekly for six weeks with one week of no dosing (seven week course) for four courses did not cause excess mortality in rats. Nor was dosing associated with any clinical toxicities except a dose dependant decrease in body weight gain and weight loss in the high dose group. So here, despite a doubling of number of courses, a dose one-half that of a dose that caused excessive mortality caused almost no toxicity. The rats were able to maintain a homeostatic compensation for the inhibition caused by pralatrexate and again we see an example of how steep is the dose response curve for pralatrexate. This compensation is possibly not related so much to diminished dTMP as the rats have such high thymine reserve, but rather their ability to maintain adequate purine synthesis in other pathways downstream of DHFR in the face of pralatrexate inhibition.

The same pattern of dose response occurs in dogs. Pralatrexate given as six weekly doses over a seven week course for two courses caused unacceptable mortality at a dose of 1 mg/kg (20 mg/m^2), but all animals survived when the dose was reduced to 0.7 mg/kg (14 mg/m^2, one female required B_{12} and folate supplementation). This is an exceptionally steep dose response curve, but again demonstrates the sharp line between effective homeostatic compensation and complete system failure. And again this is possibly due to the ability of the dogs to maintain effective purine biosynthesis in the presence of barely adequate thymine reserves. With sufficient purine biosynthesis intact, DHFR concentrations can be maintained by *de novo* synthesis.

In both rats and dogs, death was presaged by generalized deterioration of condition manifested as hunched posture, rough hair coat, weight loss, languidness and thin appearance in rats and thin appearance weight loss and inactivity in dogs. Nevertheless, in both species the most obvious clinical signs were indications of gastrointestinal toxicity. Both species showed signs of diarrhea, soft or abnormal feces and decreased food consumption; dogs showed signs of dehydration, nausea and vomiting. Despite the fact that no gross or microscopic damage was apparent in the intestine of either species, gastrointestinal damage was probably the cause of death. The loss of function of the gastrointestinal epithelium (a rapidly reproducing tissue) preceded obvious necrosis and was sufficiently severe as to cause general malnutrition and dehydration. As all other tissues were likewise poisoned death was relatively rapid once homeostasis was lost.

Other rapidly reproducing tissues were also affected, usually more so in the dog for reasons stated above. There was indication of marrow hypocellularity that correlated with decreases in red cell parameters depending on dose, but this toxicity was not limiting. Reticulocytes increased in compensation for the decreases in red cell parameters. There was spleenic enlargement and spleenic and hepatic hematopoietic brought about by compensation for marrow dysfunction and a generalized inflammatory response. In dogs thymic size and weight also increased again in compensation.

The longest study in dogs, weekly IV doses of pralatrexate as high as 0.7 mg/kg (14 mg/m^2) for a planned schedule of six weekly doses over a seven week course for seven courses is hard to compare to all the other studies. In the third week of the study there was unacceptable mortality, unlike the earlier study at the same dose. The dogs that died or were moribund showed signs of marked to severe damage to the gastrointestinal tract and the bone marrow. Instead of lowering the dose, the investigators provided dietary supplementation with canned food and with folate and B_{12} for the high dose dogs. This prevented further mortality but altered the spectrum and severity of the toxicities induced. After supplementation, dose dependant damage to the gastrointestinal tract was only mild to moderate (red discoloration of the duodenum, jejunum, ileum, cecum, colon, or rectum) while there was no further damage to the bone marrow. This is probably because in the presence of excess folate, anti-folates are displaced from DHFR six times faster than they are in the absence of folate. This study only has relevance because in the clinical studies used to establish the efficacy of pralatrexate, the diet of patients was also supplemented with both folate and B_{12}. But the high dose in this study is only half that of the clinical dose (30 mg/m^2 on the same schedule) suggesting that the supplementation shifted the toxic dose response curve dramatically. Absent supplementation, the proposed clinical dose would probably be lethal to some patients. The current clinical studies only establish the safety of pralatrexate treatment when it is given with concomitant folate and B_{12}.

In this long-term study in dogs there were some toxicities not seen as clearly elsewhere. This was the only study to demonstrate a decrease in WBC and lymphocytes, as much as 50% in high dose animals. Basophiles and eosinophils followed suit, indicating increased damage to white cell production in the marrow. Several parameters (mild clotting dysfunction, changes in AST or ALT, decreased A/G ratio) suggested minor hepatic dysfunction. Increases of 10 to 20% creatinine were consistent with decreases in kidney weights. With folate supplementation, gastrointestinal toxicity was no longer limiting; other systems then bare the brunt of pralatrexate toxicity. Nevertheless, the gastrointestinal system was the site of the majority of microscopic damage including epithelial necrosis, villus fusion, and dilation of the crypt. A decrease in absolute heart weight and the heart-to-brain weight ratio and the lack of complete resolution of this decrease during recovery suggest the possibility long term cardiac toxicity despite the lack of changes in ECGs. This is possibly due to a decreased ability of the heart to effectively repair damage from natural ware as effectively as it would in the absence of pralatrexate. Most of the other toxicities associated with pralatrexate were reversible.

The table below shows that most of the toxicities observed clinically correlated with toxicities seen in non-clinical studies. The ones that had no non-clinical correlate are those that are usually reported by the patient.

Correlation with Clinical Toxicities that Occurred with an Incidence Greater than One Percent
Clinical percentages from study PDX-008

Toxicity	Men	Women	Rats	Dogs
Mucosal inflammation	67%	77%	Observed as gross and microscopic damage to the intestinal mucosa	Observed as gross and microscopic damage to the intestinal mucosa
Thrombocytopenia	33%	57%	Not observed	Elevated about 25% in long term study
Anemia	29%	46%	Observed in all studies	Observed in all studies
Edema	26%	37%	Observed incidentally	Observed incidentally
Neutropenia	22%	29%	Not clearly observed	Observed in all studies
Anorexia	17%	11%	Observed in all studies	Observed in all studies
Hypokalemia	13%	20%	Not observed	Not observed
Pruritus	12%	20%	Normally unobservable	Normally unobservable
Liver Function Test Abnormality	11%	17%	Not observed	Observed in primary study
Leukopenia	9%	14%	Observed as marrow hypocellularity with spleenic and hepatic hematopoiesis	Observed in primary study
Dyspepsia	7%	14%	Not observed	Not observed
Dry mouth	5%	3%	Normally unobservable	Observed as dehydration
Odynophagia	1%	3%	Normally unobservable	Normally unobservable

The sponsor also studied the toxicity of (b) (4) in order to qualify the concentrations of this impurity that remain in the drug product. Dogs dosed intravenously with doses 3, 10 or 30 mg/kg (60, 200 or 600 mg/m^2) weekly for six weeks all vomited or showed signs of nausea shortly after dosing. The time of onset suggests that this is an acute neurotoxicity. There were also some signs of renal toxicity. Nevertheless, the doses in this study were sufficiently high to qualify (b) (4) at the concentrations greater than the proposed drug product specifications.

Reproductive Toxicity

In range finding studies, doses of 36 mg/m^2 in rabbits or 0.6 mg/m^2 in rats caused total liter resorption in most animals. Thus, the high doses in both non-range-finding reproductive toxicology studies were relatively low, 0.06 mg/kg/day in rats (0.36 mg/m^2/day or about 1.2% of the single clinical dose on a mg/m^2 basis) and 1 mg/kg/day in rabbits (12 mg/m^2/day or about 40% of the single clinical dose). Neither study assessed the level of systemic exposure (AUC). These doses caused only minor systemic maternal toxicity manifested as small decreases in weight gain (8% or less relative to controls). There were no other apparent maternal toxicities. Nevertheless, these doses did severely reduce fetal viability. Three high dose rabbits (of 25) aborted their litters by gestation day 25 and 8 had totally resorbed litters at necropsy. Both species showed an increase in total resorptions (late and early in the rat and early in the rabbit). There were also dose dependant increases in percent post implantation loss and total post implantation loss that were statistically significant in the high dose groups. The mean number of live fetuses decreased with increasing dose. Mean fetal weight parameters decreased with increasing dose; these decreases were significant in the HD groups.

In neither rats nor rabbits was there an increase in morphological defects among fetuses treated with pralatrexate compared to controls but in both cases the number of fetuses was significantly diminished (severely in the rabbit).

JM DeSesso and RL Jordan identified methotrexate as a teratogen as early as 1977 (Teratology. 1977 Apr;15(2):199-211). They demonstrated that methotrexate "produced cleft palate, hydrocephalus, and fore- and hindlimb reduction defects. Histological analysis revealed pyknosis and edema in mesenchymal tissues at four to eight hours following treatment. The apical ectodermal ridges (AER) of treated embryos permanently lost their characteristic pseudostratified organization." Subsequently JM DeSesso (Teratology. 1981 Apr; 23(2):197-215) demonstrated that methotrexate causes changes in cellular morphology of forelimb-buds in fetal rabbits when given to the pregnant does at relatively high single doses (19.2 mg/kg) on day 12 of gestation. He found that methotrexate caused "an increase in cytoplasmic lipid, angular contours of nuclei, but relatively little cellular death. The most prominent change was a disruption of the normal mesenchymal architecture characterized by greatly increased intercellular space and a consequent reduction in the number of intercellular junctions. Nearly normal cyto-architecture was restored by 32 hours." He concluded that methotrexate "caused disruption of intercellular contacts and possible breakdown of intercellular communication." Pralatrexate is probably teratogenic, but the fetal loss was so great in these studies that this toxicity was not detectable at these doses.

The methotrexate is labeled as a pregnancy category X and has the following text in a Black Box Warning:

"Methotrexate has been reported to cause fetal death and/or congenital anomalies. Therefore, it is not recommended for women of childbearing potential unless there is clear medical evidence that the benefits can be expected to outweigh the considered risks. Pregnant women with psoriasis or rheumatoid arthritis should not receive methotrexate."

Additionally, methotrexate is teratogenic in humans (M.E. Lloyd *et al.*, *QJM.* 1999 Oct;92(10):551-63). As pralatrexate works by the same mechanism as methotrexate it is reasonable to suspect that it might be a teratogen despite the results of the two reproductive toxicity studies. It is certainly fetotoxic and an abortifacient. The exquisite sensitivity of the developing fetus to folate deficiency is very well established.

Genotoxicity

Pralatrexate caused no increase in the incidence of reverse mutation in the *Salmonella Typhimurium* or *Escherichia coli* strains with and without metabolic activation. Bacteria are insensitive to the mutagenic effects of methotrexate (D. Clive *et al. Mutagenesis* 1990, 5(2):191-197) so this result is not surprising.

Pralatrexate did not cause an increase in chromosomal aberrations in Chinese hamster ovary cells *in vitro* at concentrations between 0.5 and 2.1 μM in the absence or presence of S9 after three hours exposure (18 hour incubation). Also, after exposure for 18 hours to concentrations between 0.0063 and 0.027 μM, pralatrexate caused no apparent increase in the percentage of cells with aberrations.

The Chromosomal Aberration test with Chinese hamster ovary cells tests for clastogenicity. Pralatrexate (and likewise methotrexate) do not interact directly with DNA but rather cause mutations indirectly. The paucity of available thymidine causes inappropriate substitutions during DNA synthesis leading to mutations and chromosome breaks. A. H. Borchers *et al.* (*Cancer Res.* 1990 Mar 15; **50**(6):1786-9) have shown that methotrexate inhibits DNA repair in the presence of other mutagens (UV radiation and ethylmethanesulfonate) in CHO cells as measured by the accumulation of single strand breaks. But, methotrexate alone in this system had little effect even after 18 hours exposure. The results of this paper also suggest the reason for the lack of response in bacterial genotoxicity tests of methotrexate. The DNA repair

mechanisms of bacteria are very different from those of mammalian cells. D. Clive *et al.* (*Mutagenesis* 1990, 5(2):191-197) have shown that methotrexate at a concentration of 0.5 μg/mL (1.1 μM, 4 hour incubation) causes a 10 fold increase in the mutation frequency over control in L5178Y/$tk^{+/-}$ mouse lymphoma cells. These investigators report that the number of mutations at the *tk* locus is about 30 times higher than it is at the *hprt* locus. Thus, the *tk* mouse lymphoma assay would probably have much more effectively demonstrated the mutagenic potential of pralatrexate *in vitro*.

In mice, doses as high as 300 mg/m^2 daily for three days caused almost complete myelosuppression; these doses were too high to test for mutagenicity by the micronucleus test. Lower doses at the same schedule (0.375 to 15 mg/m^2) caused a dose dependant decrease in the percent of polychromatic erythrocytes (PCEs) with an IC_{50} of about 9 mg/m^2 in both males and females. These doses caused only minor systemic toxicity evidenced by mild weight loss over 72 hours. Doses of 9 mg/m^2 did not cause an increase in micronucleated PCE in either males or females.

In one of the earliest papers to demonstrate the usefulness of the mouse micronucleus test P. Maier and W. Schmid (*Mutat Res.* 1976 Nov;**40**(4):325-37) used methotrexate and nine other known mutagens to demonstrate the utility of the test. The test was positive for methotrexate but the authors advise "If arrest of the cell cycle occurs, as in the case of anti-metabolites MTX and 5-FU particularly, the routine scheme of investigation needs to be modified since micronucleated cells appear only after release of the metabolic block, i.e. after a delay of 24 h." R. Albanese (*Mutat Res.* 1987 Dec; 182 (6):309-21) also used methotrexate as a test compound to demonstrate the utility of the rat micronucleus test. The Collaborative Study Group for the Micronucleus Test, the Mammalian Mutagenesis Study Group of the Environmental Mutagen Society, Japan (*Mutat Res.* 1990 Jun-Aug;234(3-4):205-22) evaluated the value of multiple doses in the mouse micronucleus test. The group prepared this monograph to standardize the test in Japan. The paper states that methotrexate "an inhibitor of nucleotide synthesis, was a weak micronucleus inducer after single dosing, but clearly positive after multiple dosing." It further states "[t]he detection spectrum would become wider if specimens are also prepared 48 h after the second dosing for metabolic inhibitors [such as methotrexate], base analogues, and the other chemicals which might show delayed activities." H. Deng et al. (Mutagenesis 2005 20(5):351-357) determined the incidence of genetic damage as measured by the micronucleus (MN) test, the comet assay, the hprt gene mutation assay and the TCR gene mutation assay in 21 workers from a plant producing methotrexate compared to 21 matched controls. The results of the MN test showed that the mean micronuclei rate (MNR) and mean micronucleated cell rate (MCR) in workers were 10 ± 1 % and 8.0 ± 0.8 %, respectively, which were significantly higher than those (5.5 ± 0.8 % and 4.4 ± 0.6 %) in controls ($p < 0.01$). In the comet assay, the mean tail length (MTL) of workers and controls were 1.30 ± 0.06 μm and 0.07 ± 0.01 μm, respectively ($p < 0.01$). The results of hprt gene mutation assay showed that the average mutation frequency (Mf-hprt) of hprt in workers was 1.00 ± 0.02 %, while that in controls was 0.86 ± 0.01 % ($p < 0.01$). In the TCR gene mutation assay the gene mutation frequencies of workers was $6.9 \pm 0.5 \times 10^{-4}$ and $1.7 \pm 0.1 \times 10^{-4}$ ($p < 0.01$). These results suggest that occupational exposure to methotrexate is associated with genetic damage. GENE-TOX, a project of TOXNET (http://toxnet.nlm.nih.gov) lists 11 studies that demonstrate the genotoxicity of methotrexate. Since the mechanism of action of pralatrexate is identical to that of methotrexate, for it to not to be genotoxic would be anomalous. One possible reason the micronucleus test was negative could be that the three day dosing caused complete growth stasis, again in this test the cells of the marrow were prevented from entering S-phase and this block was not released buy the time of the 24 hour scoring.

Unresolved toxicology issues:

None

Application Type/Number	Submission Type/Number	Submitter Name	Product Name
NDA-22468	ORIG-1	ALLOS THERAPEUTICS INC	FOLOTYN

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

WILLIAM D MCGUINN
09/04/2009

SANDI L VERBOIS
09/04/2009