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Research Article

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Bacterial Reverse Mutation Test of 2-(3-hydroxyadamantan-1-yl)-1-imino-hexahydro-pyrrolo [1,2-a] pyrazin-4-one (Vildagliptin cyclic amidine impurity) using Salmonella Typhimurium and Escherichia Coli

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Abstract

The Ames Salmonella/microsome mutagenicity assay (Salmonella test; Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations. In this study, we are going to investigate the mutagenic potential of 2-(3-hydroxy-adamantan-1-yl)-1-iminohexahydro-pyrrolo[1,2-a] pyrazin-4-one (Vildagliptin cyclic amidine impurity) using Salmonella typhimurium and Escherichia coli tester strains. The evaluation was conducted to assess point mutations at the histidine locus in four strains of Salmonella typhimurium and the tryptophan locus in E. coli WP2uvrA. The experiment employed the plate incorporation method, both with and without a metabolic activation system (S9). Cytotoxicity was assessed by measuring the thinning of the bacterial lawn and the reduction in revertant colony counts compared to vehicle controls. Results indicated no significant cytotoxic effects or mutagenic activity across tested concentrations (15.8 to 5000 µg/plate) in both the presence (10% and 20%) and absence of metabolic activation. The findings demonstrated that Vildagliptin cyclic amidine impurity did not induce point mutations in the selected bacterial strains, highlighting the lack of mutagenic potential of the compound within the tested parameters.

Keywords: AMES Assay, Bacterial Strains, Revertant colony counts, Preliminary Cytotoxicity, Mutagenic Assay, Confirmatory Assay

1. Introduction

The bacterial reverse mutation test, sometimes known as the Ames test because it was created by Bruce Ames in the early 1970s, is the most widely used assay to evaluate chemically induced gene mutation. A variety of Salmonella typhimurium and Escherichia coli bacterial strains are used in this short-term assay, which is especially intended to detect a wide range of deoxyribonucleic acid (DNA) reactive compounds that cause fixed gene mutation. In order to determine the mutagenic potential of novel chemical entities, active pharmaceutical components, or possible genotoxic contaminants in pharmaceutical products, as well as the potential for genotoxic carcinogenicity, the Ames test is widely used throughout the world as a hazard screen because there is a high predictive value for rodent carcinogenicity when a mutagenic response is obtained.

To identify potential carcinogens and mutagens in mammals, the bacterial reverse mutation assay is commonly used as an initial screening method to assess the mutagenic activity of chemicals. This assay works by detecting mutations in bacterial strains through reversion, where the bacteria regain the ability to synthesize a crucial amino acid they previously could not produce. This enables their growth in the absence of the required amino acid. Most chemicals themselves are not inherently mutagenic or carcinogenic but undergo metabolic transformation, primarily facilitated by liver enzyme systems in vivo. Therefore, testing is performed both directly and with an exogenous metabolic activation system. The most widely used method involves a co-factor-supplemented post-mitochondrial fraction (S9), derived from the livers of rats pre-treated with enzyme-inducing substances like β-Naphthoflavone and sodium phenobarbital. The his+/trp+ revertants appear as

distinct colonies against the minimal background growth of his-/trp- cells.

2. Materials & Methods

2.1 Characterization of the Bacterial Strains

Molecular Toxicology, Inc., 157, Industrial Park, Dr. Boone, NC 28607, provided four test cultures of histidine-dependent Salmonella typhimurium (histidine auxotrophs) and one strain of tryptophan-dependent Escherichia coli (tryptophan auxotrophs). The test used working stocks made from master vials (frozen permanents made from culture discs) that were kept as frozen stocks in a deep freezer at -70 ± 10°C.

Tester Strains Genotypes				
Tester Strains	***his/trp* Mutation**	**Additional Mutations**		**Plasmid**
		Repair	**LPS**	
TA98	*His*D3052	*uvr*B	*Rfa*	pKM101
TA100	*His*G46	*uvr*B	*Rfa*	pKM101
TA1535	*His*G46	*uvr*B	*Rfa*	-
TA1537	*His*C3076	*uvr*B	*Rfa*	-
E. coli WP2*uvr*A	*trp*E	*uvr*A	-	-

The revertants were identified as colonies that grew in trace quantities of histidine & biotin (Salmonella typhimurium strains) and tryptophan (E. coli WP2uvrA strain). Frame shift and base pair substitution mutations are represented to identify mutagens of both types. Additional genetic markers (uvrB, rfa mutation, pKM101 plasmid) enhance the sensitivity of the strains to certain types of mutagens.

Genotype Markers	**Criteria**
Histidine-Biotin Dependency	Growth on Histidine-Biotin plus plates. No growth on His/Bio minus plates.
Histidine dependency	No Growth on Histidine minus plates.
Biotin Dependency	No Growth on Biotin minus plates
Tryptophan dependency (*E. coli* WP2*uvr*A)	Growth on Tryptophan plus plates No Growth on Tryptophan minus plates
**rfa* Mutation (Crystal Violet Sensitivity)	Zone of inhibition around the crystal violet disc with strain bearing *rfa* mutation.
**uvr*A Mutation	No growth on the UV irradiated region of plates containing *E. coli* WP2*uvr*A.
**uvr*B Mutation	No growth on the UV irradiated region of plates containing strains TA98, TA100, TA1535 and TA1537.
*Presence of Plasmid pKM101 (Ampicillin resistance)	No growth for strains TA1535 and TA1537. Growth on plates for strains TA98, TA100.

2.2 Preparation of Overnight Culture

Frozen stock cultures were inoculated in a flask containing 25 ml of Oxoid nutrient broth No. 2. Following inoculation, the flasks were placed in shaking water bath at 37 ± 2 °C shaking at 120 ± 10 rpm for 15:15 and 14:23 (hh:mm) in the preliminary cytotoxicity and mutagenicity assay respectively. Bacterial cultures removed from the shaker water bath at the end of the incubation period were held at 2 - 8°C (cold room) until use on the same day.

2.3 Bacterial Cell Viability

The bacterial cell viability was determined by measuring the optical density of the nutrient broth containing cells at 670 nm using a spectrophotometer. The OD value of all the tester strains were in the acceptable range i.e., between 0.2 and 0.6 thus ensuring the cell density of at least 1 x 109 CFU/ml.

3. Details of Media

- **Culture Broth**

Oxoid Nutrient Broth No. 2 (2.5 % w/v) was used to grow overnight cultures of the tester strains.

- **Minimal Glucose Agar Plates**

Vogel-Bonner minimal medium E supplemented with 1.5% w/v bacto agar and 1.0 % (w/v) glucose was used as bottom agar/Minimal Glucose Agar (MGA).

- **Top Agar for Selection of Revertants**

Top (overlay) agar was prepared with 0.6 % (w/v) bacto agar and 0.6 % (w/v) sodium chloride and supplemented with 10 ml of 0.5 mM histidine/biotin (for Salmonella typhimurium strains) or 0.1 mM tryptophan solution (for Escherichia coli strains) per 100 ml of top agar for selection of histidine/tryptophan revertants.

4. Preparation of S9 Mix

S9 mix (cofactors and liver homogenate) was prepared freshly prior to use. In the preliminary cytotoxicity assay 2.5 mL of liver homogenate was added to 22.5 mL of cofactor mix to achieve 10 % v/v. In the Mutagenicity Assay, 6 mL of liver homogenate was added to 54 mL of cofactor mix to achieve 10 % v/v. In the Confirmatory Assay, 14 mL of liver homogenate was added to 56 ml of cofactor mix to achieve the concentration of 20 % v/v.

5. Method of Formulation Preparation

In the preliminary cytotoxicity assay, 267.5 mg of test item was dissolved in Sterile water and made up to 5 mL and mixed with aid of vortex mixer which was equivalent to the concentration of 50 mg/mL. From this, subsequent dilutions were made with sterile water to achieve concentrations of 15.8, 5.0, 1.58, 0.5 and 0.158 mg/ml and each dose spaced by dilution factor of 3.16. For mutagenicity and confirmatory assays, 642.0 mg of test item was dissolved in sterile water mixed with the aid of vortex mixer for complete dissolution and then final volume was made upto 10 ml using volumetric flask and additional 2 ml was added using micropipette to get the final volume of 12mL. This was equivalent to a concentration of 50 mg/mL. Subsequently, serial dilutions were made with spacing factor 2 using vehicle- Sterile water from its immediate higher dose to achieve concentrations of 25, 12.5, 6.25, and 3.125 mg/mL. Formulations were mixed thoroughly with vortex mixer, during stock preparation, dilutions and treatment.

6. Dose Concentration Analysis

0.5 ml of samples each from 50 mg/ml, 3.125 mg/ml, and vehicle were subjected to dose formulation analysis. The formulations were found to be stable up to 48 hours at room temperature as per Analytical method validation. Results of dose concentration analysis were tabulated in Table 4.

• Preparation of Ortho Phosphoric Solution

Transferred 6.9 ml of orthophosphoric acid into a 100 mL volumetric flask containing 50 ml of water, diluted to volume with water and mixed well.

• Preparation of Buffer Solution

Weighed and transferred 5.44305 g of Potassium dihydrogen phosphate, 17.41844 g of Dipotassium hydrogen phosphate anhydrous into a 2liters mobile phase bottle containing 2000 mL water, dissolved and adjusted pH of the solution to 7.205 with dilute ortho-phosphoric acid solution. Filtered the solution through 0.45 µm membrane filter and degassed.

• Preparation of Mobile Phase-A

Mixed 1200 mL of Buffer solution, 1800 mL of water, 150 mL of Acetonitrile and 150 mL of methanol in the ratio of 40:60:5:5 (% v/v) respectively sonicated to degas.

• Preparation of Mobile Phase-B

Mixed 800mL of Buffer solution, 900 mL of Acetonitrile and 300 mL of methanol in the ratio of 40:45:15 (%v/v) respectively sonicated to degas.

• Preparation of Diluent and Blank Solution

Mobile phase A was used as Diluent and Blank solution.

• Preparation of Standard Stock Solution A

10.84 mg of Vildagliptin Cyclic Amidine Impurity standard was weighed and transferred into a 50 ml volumetric flask. Added diluent, sonicated to dissolve and diluted to volume with diluent and mixed well.

• Preparation of Standard Solution (100% Target Concentration)

A 1.0 ml of Vildagliptin Cyclic Amidine Impurity Standard stock solution was be transferred into a 10 ml volumetric flask, added diluent, mixed and made up to volume with diluent.

• Preparation of Sample Solution for VC - 0.0 mg/ml

Dose formulation solution 0.345 ml of was transferred into a 50 ml volumetric flask and volume was made up to volume with diluent.

• Preparation of Sample Solution for F1-50.0 mg/ml

Dose formulation solution 0.215 ml was transferred into a 100.0 ml volumetric flask and volume was made up to volume with diluent. Further 2.0 ml was transferred into a 10.0 ml volumetric flask and volume was made up to volume with diluent.

• Preparation of Sample Solution for F5-3.125 mg/ml

Dose formulation solution 0.345 ml of was transferred into a 50 ml volumetric flask and volume was made up to volume with diluent.

7. Preliminary Cytotoxicity Assay

The preliminary cytotoxicity assay was performed by plate incorporation method, in tester strains TA98 and TA100 both in the presence (10%) and absence of metabolic activation system. The tester strains were exposed to the test item concentrations of 15.8, 50, 158, 500, 1580 and 5000 µg/plate both in the presence (10%) and absence of metabolic activation system. During the treatment, 2 ml of molten top agar (maintained at 47 °C) containing trace quantity of histidine and biotin (Salmonella strains), 500 µl of S9 mix for with-S9 or 0.1 mM sodium phosphate buffer for without-S9, 100 µl of respective tester strain along with either the vehicle or test item or positive control formulations were added in sterile tubes. The contents were mixed and overlaid onto the surface of minimal glucose agar plates. No Precipitation of test item was observed both in the presence and absence of metabolic activation system in both the strains. Following treatment, all treated plates were incubated at 37ºC for 68:18 (hh:mm). Following incubation, all the plates were observed under microscope for background bacterial lawn inhibition and presence of precipitation on the plates. The bacterial background lawn was evaluated for the evidence of cytotoxicity. Also, cytotoxicity was scored relative to the vehicle control and recorded along with the mean revertant counts for each treatment. Revertant colonies were counted by automated colony counter.

8. Mutagenicity Assay

The mutagenicity assay was performed using the tester strains TA1537, TA1535, TA98, TA100 and E. coli WP2uvrA both in the

presence (10% v/v S9) and absence of metabolic activation system. Based on the solubility, precipitation and preliminary cytotoxicity assay results, the mutagenicity assay was performed using test item concentrations of 5000, 2500, 1250, 625 and 312.5 µg/plate both in the presence (10% v/v) and absence of metabolic activation system. In mutagenicity assay, the tester strains were exposed to the test item via plate incorporation method, in which, 2 ml of molten top agar (maintained at 47°C) containing trace quantity of histidine and biotin (for Salmonella typhimurium strains)/ tryptophan (for Escherichia coli strains), 500 µl of S9 mix (10% S9) for with S9 or 0.1 mM sodium phosphate buffer for without S9, 100 µl of respective tester strain along with either the vehicle or test item or positive control formulations were added in sterile tubes. The contents were mixed and overlaid onto the surface of minimal glucose agar plates. No Precipitation of test item was observed from both in the presence and absence of metabolic activation system in all five tester strains.

9. Confirmatory Assay

Confirmatory assay was performed using test item concentrations of 5000, 2500, 1250, 625 and 312.5 µg/plate in the presence (20% v/v) of metabolic activation system. Tester strains were exposed to the test item via plate incorporation method, in which, 2 ml of molten top agar (maintained at 47°C) with trace quantity of histidine and biotin (for Salmonella typhimurium strains)/ tryptophan (for Escherichia coli strains), 500 µl of S9 mix (10% S9), 100 µl each of the tester strain and either the vehicle or test item or positive control formulations were added in sterile tubes, the contents were mixed and overlaid onto the surface of minimal glucose agar plates. No Precipitation of test item was observed at 312.5 to 5000 µg/plate concentrations. All the mutagenicity and confirmatory assay treated plates were incubated at 37 ºC for 65:17 (hh:mm). Following incubation, all the plates were observed under microscope for background lawn inhibition, presence of precipitation on the plates and were recorded along with the individual revertant counts for each test concentration. Revertant colonies were counted by automated colony counter.

10. Assay Evaluation Criteria

No formal hypothesis testing was performed to analyse the data. The responses observed in the assay were evaluated as per the criteria mentioned below.

For the test item to be considered positive, the test item must produce at least a 2- fold increase in the mean revertants per plate of at least one of these tester strains; TA98, TA100 and E. coli WP2uvrA over the mean revertants per plate of respective vehicle and; at least a 3-fold increase in the mean revertants per plate in one or both of the tester strains TA1535 and TA1537 in comparison with appropriate vehicle control. This increase in the mean number of revertants colony units per plate can be accompanied by a dose response (in at least 2-3 consecutive concentrations) to increasing concentrations of the test item.

11. Results

11.1 Preliminary Cytotoxicity Assay (Refer Table 1; Appendix 1, 4)

Cytotoxicity was evaluated by examining the thinning of the background bacterial lawn and the decrease in revertant colony counts relative to the vehicle control. No signs of toxicity, indicated by thinning of the background bacterial lawn and a decrease in revertant count, were noticed at 15. 8, 50, 158, 500, 1580, and 5000 µg/plate, in comparison to the vehicle control. The test item at the specified concentrations did not produce a significant reduction in revertant colony counts, both with (10%) and without the metabolic activation system, in both tester strains TA98 and TA100 compared to their respective vehicle control. According to the initial cytotoxicity assay findings, 5000 µg/plate was chosen as the highest concentration for the mutagenicity assay, both with (10%) and without the metabolic activation system, as well as for the confirmatory assay with (20%) metabolic activation. The subsequent lower concentrations designated were 2500, 1250, 625, and 312. 5 µg/plate. No precipitation was detected from 15. 8 µg/plate to 5000 µg/plate in both the TA98 and TA100 strains, regardless of the presence or absence of the metabolic activation system.

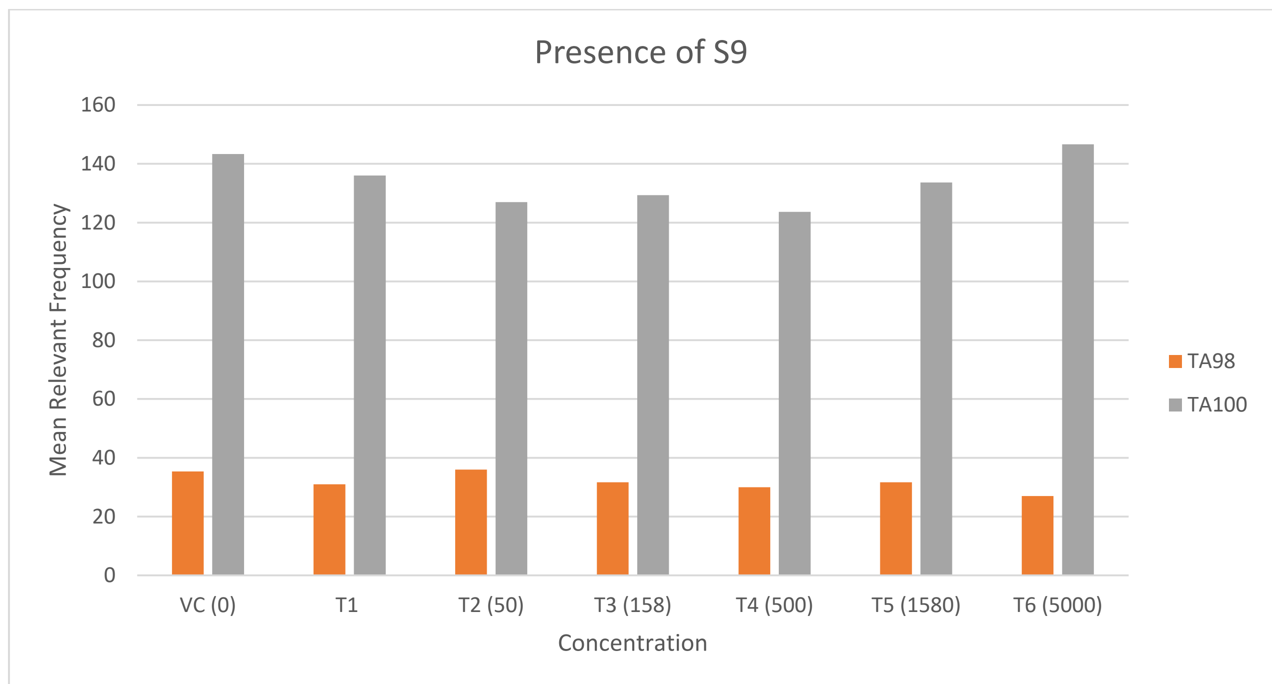

Figure 1: Diagrammatic representation of Preliminary Cytotoxicity Assay - Mean Revertant Frequency (Presence of S9)

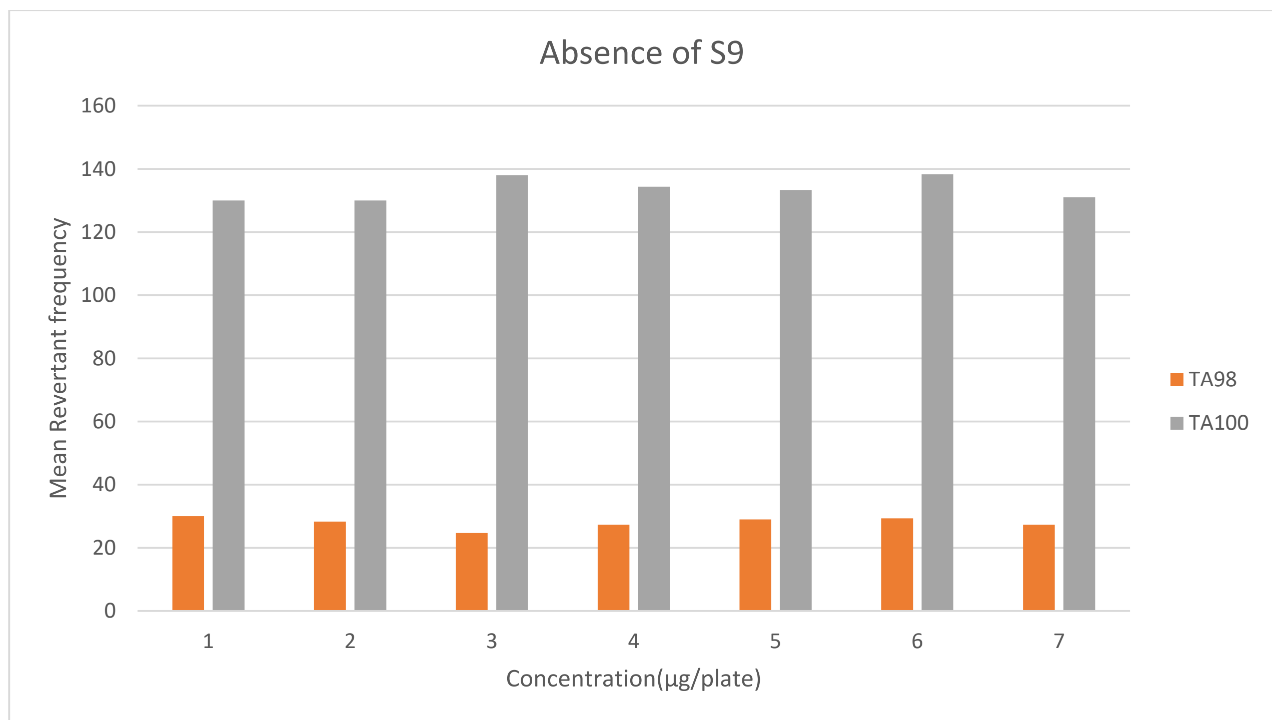

Figure 2: Diagrammatic representation of Preliminary Cytotoxicity Assay - Mean Revertant Frequency (Absence of S9)

11.2 Mutagenicity Assay (Refer Table 2; Appendix 2, 5)

The mean concentration of the test item found in the highest and lowest tested dose formulations prepared for treatment were 51.0631 mg/mL and 3.1250 mg/mL against the nominal concentrations of 50 mg/mL and 3.125 mg/mL respectively. The results fell within 100 ± 10 % of respective nominal concentrations, thereby meeting the acceptance criteria. Test item treated at concentrations from 312.5 to 5000 µg/plate did not elicit any considerable increase in the mean revertant colony counts both in the presence (10%) and absence of metabolic activation system in TA1537, TA1535,

TA98, TA100 and E.coli WP2uvrA tester strains in comparison to the vehicle control. No precipitation of test item was observed on plates tested at 312.5 to 5000 µg/plate in all five tester strains. No toxicity in the form of thinning of background bacterial lawn was observed in TA1537, TA1535, TA98, TA100 and E.coli WP2uvrA treated at 312.5 to 5000 µg/plate in presence (10%) and absence of metabolic activation system.

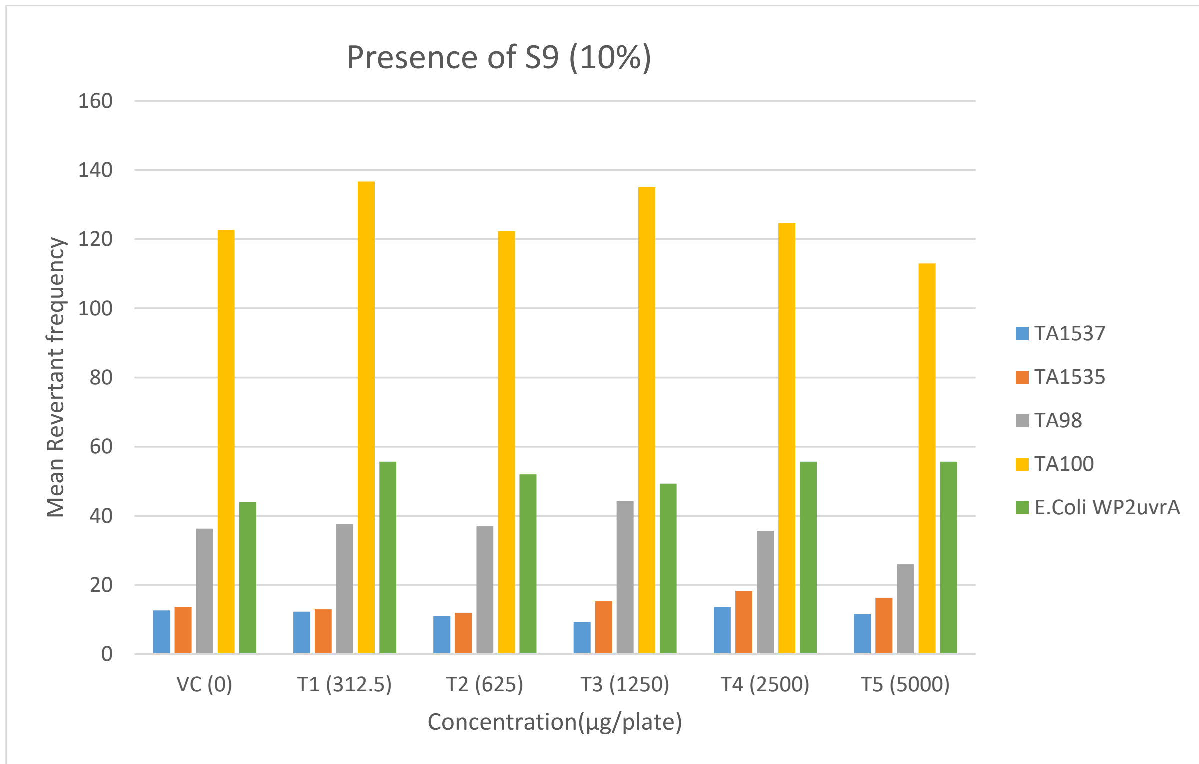

Figure 3: Diagrammatic representation of Mutagenicity Assay - Mean Revertant Frequency Metabolic Activation: Presence of S9 (10%)

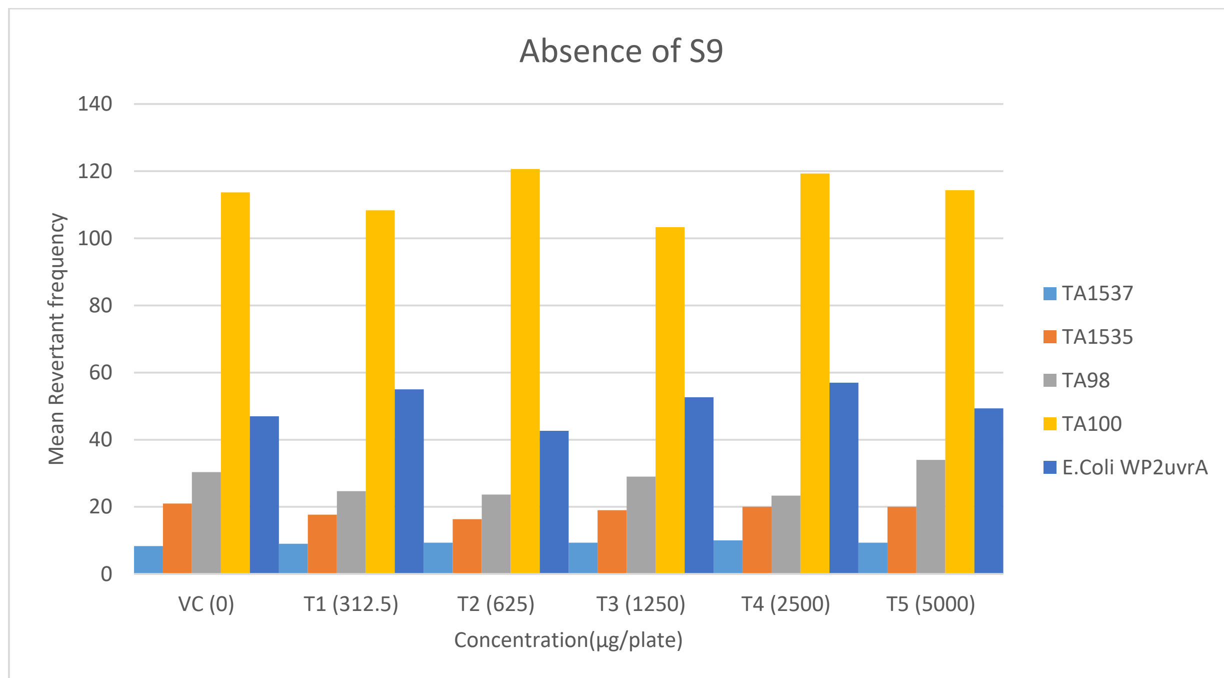

Figure 4: Diagrammatic representation of Mutagenicity Assay Mean Revertant Frequency Metabolic Activation: Absence of S9

11.3 Confirmatory Assay (Refer Table 3; Appendix 3, 6)
Test item treated at concentrations from 312.5 to 5000 µg/plate did not elicit any considerable increase in the mean revertant colony counts in the presence (20%) of metabolic activation system in TA1537, TA1535, TA98, TA100 and E. coli WP2uvrA tester strains in comparison to the vehicle control. No precipitation of test item was observed on plates tested at 312.5 to 5000 µg/plate in all five tester strains. No toxicity in the form of thinning of background bacterial lawn was observed in TA1537, TA1535, TA98, TA100 and E. coli WP2uvrA treated at 312.5 to 5000 µg/plate in presence (20%) of metabolic activation system. The mean spontaneous revertant colony count frequency of vehicle controls fell within the range of the respective in-house historical control data. The positive control treated plates exhibited multi-fold increase (>3 fold) in the mean revertant colonies (his+/ trp+) respective to their strains indicating the sensitivity of the test system towards their specific mutagens and confirmed that the test conditions adopted were appropriate; and that the metabolic activation system functioned properly.

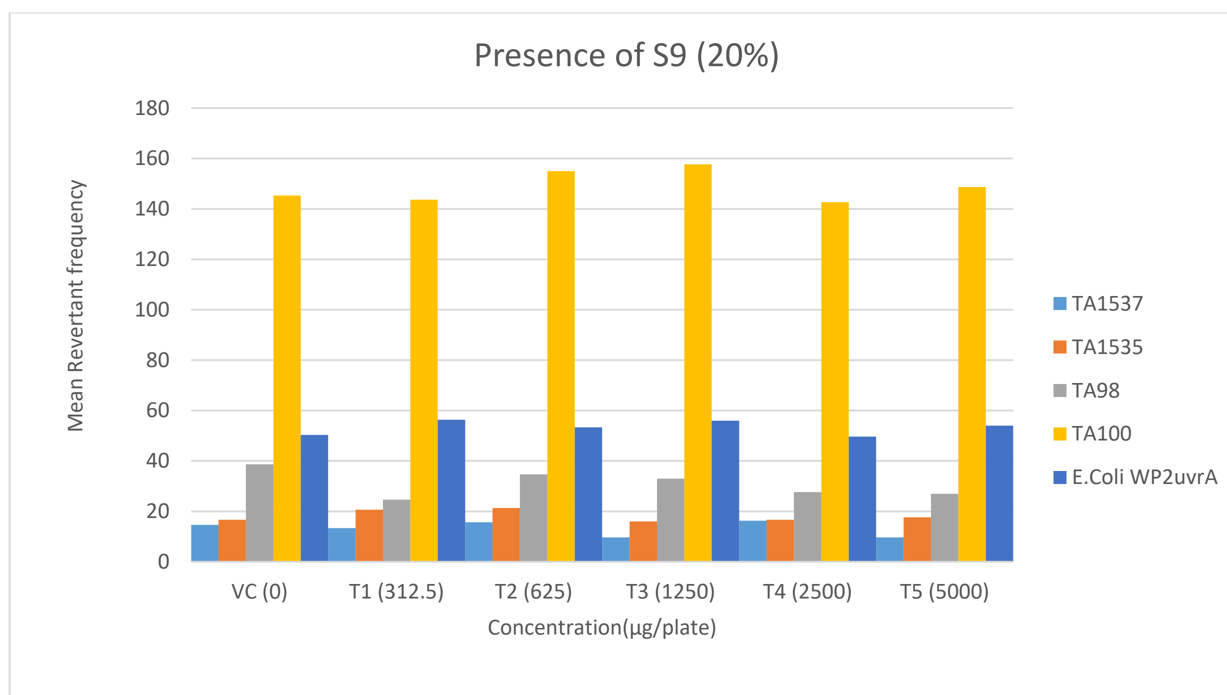

Figure 5: Diagrammatic representation of Confirmatory Assay - Mean Revertant Frequency Metabolic Activation: Presence of S9 (20%)

12. Conclusion
Based on the results, it is concluded 2-(3-hydroxy-adamantan-1-yl)-1-imino-hexahydropyrrolo[1,2-a] pyrazin-4-one (Vildagliptin cyclic amidine impurity) tested up to 5000 µg/plate under the tested conditions, is non-mutagenic in Bacterial Reverse Mutation Test.

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Treatment Code & Test Concentration (µg/plate)	Presence of S9 (10%)					
	TA98			TA100		
	Mean	SD	#	Mean	SD	#
VC (0)	35.33	1.53	NA	143.33	15.50	NA
T1 (15.8)	31.00	1.73	0.88	136.00	13.75	0.95
T2 (50)	36.00	4.00	1.02	127.00	5.20	0.89
T3 (158)	31.67	1.53	0.90	129.33	2.08	0.90
T4 (500)	30.00	3.00	0.85	123.67	2.08	0.86
T5 (1580)	31.67	2.08	0.90	133.67	11.02	0.93
T6 (5000)	27.00	1.73	0.76	146.67	4.04	1.02
PC-2AA (2.5)	1020.00	20.22	28.87	1150.00	47.15	8.02

Treatment Code & Test Concentration (µg/plate)	Absence of S9					
	TA98			TA100		
	Mean	SD	#	Mean	SD	#
VC (0)	30.00	2.00	NA	130.00	2.00	NA
T1 (15.8)	28.33	3.06	0.94	130.00	5.20	1.00
T2 (50)	24.67	4.51	0.82	138.00	6.24	1.06
T3 (158)	27.33	1.53	0.91	134.33	1.53	1.03
T4 (500)	29.00	5.29	0.97	133.33	5.86	1.03
T5 (1580)	29.33	3.51	0.98	138.33	3.06	1.06
T6 (5000)	27.33	0.58	0.91	131.00	9.64	1.01
PC-2NF (2.5)	187.67	9.07	6.26	-	-	-
PC-SA (3.0)	-	-	-	749.67	40.50	5.77

Table 1: Preliminary Cytotoxicity Assay - Mean Revertant Frequency

Treatment Code & Test Concentration (µg/plate)	TA1537			TA1535			TA98		
	Mean	SD	#	Mean	SD	#	Mean	SD	#
VC (0)	12.67	2.89	NA	13.67	8.08	NA	36.33	2.52	NA
T1 (312.5)	12.33	1.53	0.97	13.00	2.65	0.95	37.67	8.39	1.04
T2 (625)	11.00	1.00	0.87	12.00	1.00	0.88	37.00	9.54	1.02
T3 (1250)	9.33	2.31	0.74	15.33	5.03	1.12	44.33	13.01	1.22
T4 (2500)	13.67	4.16	1.08	18.33	1.53	1.34	35.67	6.51	0.98
T5 (5000)	11.67	3.21	0.92	16.33	2.52	1.19	26.00	13.45	0.72
PC-2AA (2.5)	174.33	20.13	13.76	258.33	27.97	18.90	807.67	19.86	22.23

Treatment Code & Test Concentration (µg/plate)	TA100			*E. coli* WP2*uvr*A		
	Mean	SD	#	Mean	SD	#
VC (0)	122.67	12.42	NA	44.00	5.57	NA
T1 (312.5)	136.67	8.74	1.11	55.67	8.96	1.27
T2 (625)	122.33	6.11	1.00	52.00	3.00	1.18
T3 (1250)	135.00	20.66	1.10	49.33	8.50	1.12
T4 (2500)	124.67	16.17	1.02	55.67	11.72	1.27
T5 (5000)	113.00	3.61	0.92	55.67	10.02	1.27
PC-2AA (2.5)	1324.33	68.24	10.80	-	-	-
PC-2AA (10.0)	-	-	-	250.00	13.11	5.68

Table 2: Mutagenicity Assay - Mean Revertant Frequency Metabolic Activation: Presence of S9 (10%)

Treatment Code & Test Concentration (µg/plate)	TA1537			TA1535			TA98		
	Mean	SD	#	Mean	SD	#	Mean	SD	#
VC (0)	8.33	3.21	NA	21.00	3.00	NA	30.33	4.16	NA
T1 (312.5)	9.00	1.73	1.08	17.67	3.79	0.84	24.67	4.93	0.81
T2 (625)	9.33	2.31	1.12	16.33	4.73	0.78	23.67	5.03	0.78
T3 (1250)	9.33	3.06	1.12	19.00	1.00	0.90	29.00	7.55	0.96
T4 (2500)	10.00	3.00	1.20	20.00	1.73	0.95	23.33	1.53	0.77
T5 (5000)	9.33	2.08	1.12	20.00	2.00	0.95	34.00	7.94	1.12
PC-ICR191 (0.5)	122.67	25.70	14.73	-	-	-	-	-	-
PC –SA (3.0)	-	-	-	690.00	19.31	32.86	-	-	-
PC-2NF (2.5)	-	-	-	-	-	-	198.67	22.14	6.55

Treatment Code & Test Concentration (µg/plate)	TA100			*E. coli* WP2*uvr*A		
	Mean	SD	#	Mean	SD	#
VC (0)	113.67	9.61	NA	47.00	7.94	NA
T1 (312.5)	108.33	4.04	0.95	55.00	5.57	1.17
T2 (625)	120.67	5.13	1.06	42.67	7.57	0.91
T3 (1250)	103.33	8.96	0.91	52.67	11.59	1.12
T4 (2500)	119.33	8.39	1.05	57.00	6.93	1.21
T5 (5000)	114.33	14.47	1.01	49.33	7.23	1.05
PC-SA (3.0)	709.00	66.51	6.24	-	-	-
PC-4NQO (1.0)	-	-	-	683.67	35.16	14.55

Table 2 (Continued): Mutagenicity Assay Mean Revertant Frequency Metabolic Activation: Absence of S9

Treatment Code & Test Concent	TA1537			TA1535			TA98		
	Mean	**SD**	**#**	**Mean**	**SD**	**#**	**Mean**	**SD**	**#**
VC (0)	14.67	4.16	NA	16.67	4.93	NA	38.67	3.51	NA
T1 (312.5)	13.33	3.21	0.91	20.67	4.04	1.24	24.67	5.86	0.64
T2 (625)	15.67	4.93	1.07	21.33	2.08	1.28	34.67	6.11	0.90
T3 (1250)	9.67	2.31	0.66	16.00	1.73	0.96	33.00	7.21	0.85
T4 (2500)	16.33	6.66	1.11	16.67	5.03	1.00	27.67	4.04	0.72
T5 (5000)	9.67	1.53	0.66	17.67	4.62	1.06	27.00	5.29	0.70
PC-2AA (2.5)	159.67	6.43	10.88	204.00	28.21	12.24	867.67	15.57	22.44

Treatment Code & Test Concent	TA100			*E. coli* WP2*uvr*A		
	Mean	**SD**	**#**	**Mean**	**SD**	**#**
VC (0)	145.33	12.42	NA	50.33	7.64	NA
T1 (312.5)	143.67	16.20	0.99	56.33	2.08	1.12
T2 (625)	155.00	5.00	1.07	53.33	7.51	1.06
T3 (1250)	157.67	3.51	1.08	56.00	11.79	1.11
T4 (2500)	142.67	11.85	0.98	49.67	3.06	0.99
T5 (5000)	148.67	19.66	1.02	54.00	13.00	1.07
PC-2AA (2.5)	877.33	52.84	6.04	-	-	-
PC-2AA (10.0)	-	-	-	208.00	7.55	4.13

Table 3: Confirmatory Assay - Mean Revertant Frequency Metabolic Activation: Presence of S9 (20%)

Group	Nominal concentration (mg/ml)	Found Concentration (mg/ml)		Average (mg/ml)	Observed Concentration (%)	Average (%)	SD	Deviation (%)
VC	0	R1	0	0	0	0	0	0
F1	50	R1	49.8166	51.0631	99.6	102.1	3.479	2.1
		R2	53.0468		106.1			
		R3	50.3258		100.7			
F5	3.125	R1	3.2779	3.125	104.9	104	0.8544	3.8
		R2	3.2469		103.9			
		R3	3.2244		103.2			

Table 4: Results of Dose Concentration Analysis

Treatment Code & Test Concentration (µg/plate)	R	Presence of S9				Absence of S9			
		TA98	TA100	TA98	TA100				
		P	**B**	**P**	**B**	**P**	**B**	**P**	**B**
VC (0)	R1	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N
T1 (15.8)	R1	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N
T2 (50)	R1	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N
T3 (158)	R1	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N
T4 (500)	R1	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N
T5 (1580)	R1	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N
T6 (5000)	R1	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N
PC-2AA (2.5)	R1	X	N	X	N	-	-	-	-
	R2	X	N	X	N	-	-	-	-
	R3	X	N	X	N	-	-	-	-
PC-SA (3.0)	R1	-	-	-	-	-	-	X	N
	R2	-	-	-	-	-	-	X	N
	R3	-	-	-	-	-	-	X	N
PC-2NF (2.5)	R1	-	-	-	-	X	N	-	-
	R2	-	-	-	-	X	N	-	-
	R3	-	-	-	-	X	N	-	-

Appendix 1: Preliminary Cytotoxicity Assay - Precipitation and Background Lawn Evaluation

Treatment Code & Test Concentration (µg/plate)	R	TA1537		TA1535		TA98		TA100		*E. coli* WP2*uvrA*	
		P	**B**	**P**	**B**	**P**	**B**	**P**	**B**	**P**	**B**
VC (0)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N

T1 (312.5)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T2 (625)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T3 (1250)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T4 (2500)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T5 (5000)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
PC-2AA (2.5)	R1	X	N	X	N	X	N	X	N	-	-
	R2	X	N	X	N	X	N	X	N	-	-
	R3	X	N	X	N	X	N	X	N	-	-
PC-2AA (10.0)	R1	-	-	-	-	-	-	-	-	X	N
	R2	-	-	-	-	-	-	-	-	X	N
	R3	-	-	-	-	-	-	-	-	X	N

Appendix 2: Mutagenicity Assay - Precipitation and Background Lawn Evaluation Metabolic Activation: Presence of S9 (10%)

Treatment Code & Test Concentration (µg/plate)	**R**	**TA1537**		**TA1535**		**TA98**		**TA100**		***E. coli***	
										WP2*uvr*A	
		P	**B**	**P**	**B**	**P**	**B**	**P**	**B**	**P**	**B**
VC (0)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T1 (312.5)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T2 (625)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T3 (1250)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T4 (2500)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T5 (5000)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N

PC-ICR191 (0.5)	R1	X	N	-	-	-	-	-	-	-	-
	R2	X	N	-	-	-	-	-	-	-	-
	R3	X	N	-	-	-	-	-	-	-	-
PC-SA (3.0)	R1	-	-	X	N	-	-	X	N	-	-
	R2	-	-	X	N	-	-	X	N	-	-
	R3	-	-	X	N	-	-	X	N	-	-
PC-2NF (2.5)	R1	-	-	-	-	X	N	-	-	-	-
	R2	-	-	-	-	X	N	-	-	-	-
	R3	-	-	-	-	X	N	-	-	-	-
PC-4NQO (1.0)	R1	-	-	-	-	-	-	-	-	X	N
	R2	-	-	-	-	-	-	-	-	X	N
	R3	-	-	-	-	-	-	-	-	X	N

Appendix 2 continued: Mutagenicity Assay - Precipitation and Background Lawn Evaluation Metabolic Activation: Absence of S9

Treatment Code & Test Concentration (µg/plate)	**R**	**TA1537**		**TA1535**		**TA98**		**TA100**		***E. coli* WP2*uvr*A**	
		P	**B**	**P**	**B**	**P**	**B**	**P**	**B**	**P**	**B**
VC (0)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T1 (312.5)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T2 (625)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T3 (1250)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T4 (2500)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
T5 (5000)	R1	X	N	X	N	X	N	X	N	X	N
	R2	X	N	X	N	X	N	X	N	X	N
	R3	X	N	X	N	X	N	X	N	X	N
PC-2AA (2.5)	R1	X	N	X	N	X	N	X	N	-	-
	R2	X	N	X	N	X	N	X	N	-	-
	R3	X	N	X	N	X	N	X	N	-	-
PC-2AA (10.0)	R1	-	-	-	-	-	-	-	-	X	N
	R2	-	-	-	-	-	-	-	-	X	N
	R3	-	-	-	-	-	-	-	-	X	N

Appendix 3: Confirmatory Assay - Precipitation and Background Lawn Evaluation Metabolic Activation: Presence of S9 (20%)

Treatment Code & Test Concentration (µg/plate)		TA98 Presence of S9			TA100 Presence of S9		
		R1	R2	R3	R1	R2	R3
VC	0	34	37	35	143	128	159
T1	15.8	30	30	33	124	151	133
T2	50	36	32	40	124	133	124
T3	158	33	32	30	131	127	130
T4	500	27	30	33	122	126	123
T5	1580	34	30	31	145	133	123
T6	5000	26	29	26	151	146	143
PC-2AA	2.5	1043	1005	1012	1183	1171	1096

Treatment Code & Test Concentration (µg/plate)		TA98 Absence of S9			TA100 Absence of S9		
		R1	R2	R3	R1	R2	R3
VC	0	32	28	30	128	130	132
T1	15.8	29	25	31	136	127	127
T2	50	29	25	20	133	136	145
T3	158	27	29	26	136	133	134
T4	500	25	27	35	131	140	129
T5	1580	26	33	29	139	141	135
T6	5000	28	27	27	127	142	124
PC-2NF	2.5	198	181	184	-	-	-
PC-SA	3	-	-	-	790	709	750

Appendix 4: Preliminary Cytotoxicity Assay - Individual Revertant Colony Counts

Treatment Code & Test Concentration (µg/plate)	TA1537			TA1535			TA98		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
VC (0)	11	16	11	9	23	9	34	36	39
T1 (312.5)	12	14	11	11	16	12	42	28	43
T2 (625)	11	12	10	13	11	12	36	47	28
T3 (1250)	8	8	12	10	20	16	45	31	57
T4 (2500)	15	17	9	18	17	20	42	29	36
T5 (5000)	14	8	13	16	14	19	41	22	15
PC-2AA (2.5)	153	193	177	290	237	248	822	785	816

Treatment Code & Test Concentration (µg/plate)	TA100			*E. coli* WP2*uvr*A		
	R1	R2	R3	R1	R2	R3
VC (0)	137	115	116	45	38	49
T1 (312.5)	127	144	139	51	50	66
T2 (625)	129	121	117	55	52	49
T3 (1250)	152	112	141	43	59	46
T4 (2500)	142	110	122	47	69	51
T5 (5000)	110	117	112	55	66	46
PC-2AA (2.5)	1382	1342	1249	-	-	-
PC-2AA (10.0)	-	-	-	236	252	262

Appendix 5: Mutagenicity Assay - Individual Revertant Colony Counts Metabolic Activation: Presence of S9 (10%)

Treatment Code & Test Concentration (µg/plate)	TA1537			TA98			TA98		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
VC (0)	12	6	7	18	21	24	27	29	35
T1 (312.5)	8	11	8	15	22	16	19	28	27
T2 (625)	12	8	8	11	20	18	19	23	29
T3 (1250)	12	6	10	18	20	19	30	36	21
T4 (2500)	10	13	7	19	22	19	25	22	23
T5 (5000)	10	7	11	18	22	20	31	43	28
PC-ICR191 (0.5)	99	150	119	-	-	-	-	-	-
PC-SA (3.0)	-	-	-	694	707	669	-	-	-
PC-2NF (2.5)	-	-	-	-	-	-	224	183	189

Treatment Code & Test Concentration (µg/plate)	TA100			*E. coli* WP2*uvr*A		
	R1	R2	R3	R1	R2	R3
VC (0)	124	112	105	38	50	53
T1 (312.5)	104	112	109	60	49	56
T2 (625)	122	115	125	48	34	46
T3 (1250)	108	93	109	47	66	45
T4 (2500)	115	114	129	61	61	49
T5 (5000)	131	107	105	41	54	53
PC-SA (3.0)	775	642	710	-	-	-
PC-4NQO (1.0)	-	-	-	696	711	644

Appendix 5 Continued: Mutagenicity Assay - Individual Revertant Colony Counts Metabolic Activation: Absence of S9

Treatment Code & Test Concentration (µg/plate)	TA1537						TA98		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
VC (0)	16	10	18	19	20	11	39	42	35
T1 (312.5)	11	12	17	20	25	17	29	27	18
T2 (625)	19	18	10	19	23	22	36	28	40
T3 (1250)	11	11	7	14	17	17	27	31	41
T4 (2500)	18	9	22	12	22	16	32	24	27
T5 (5000)	8	10	11	23	15	15	29	21	31
PC-2AA (2.5)	155	167	157	178	234	200	866	853	884

Treatment Code & Test Concentration (µg/plate)	TA100			*E. coli* WP2*uvr*A		
	R1	R2	R3	R1	R2	R3
VC (0)	131	153	152	42	52	57
T1 (312.5)	152	154	125	58	57	54
T2 (625)	150	160	155	61	46	53
T3 (1250)	161	158	154	53	46	69
T4 (2500)	129	149	150	49	53	47
T5 (5000)	159	126	161	69	47	46
PC-2AA (2.5)	922	891	819	-	-	-
PC-2AA (10.0)	-	-	-	216	201	207

Appendix 6: Confirmatory Assay - Individual Revertant Colony Counts Metabolic Activation: Presence of S9 (20%)

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