

Evaluation of Genotoxicity of CVA1020 through Ames and *in Vitro* Chromosomal Aberration Tests

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Abstract: The aim of the present study was to assess the mutagenic potential of CVA1020 using the bacterial reverse mutation assay (Ames test) with bacteria and *in vitro* chromosomal aberration test with mammalian cells. In the reverse mutation test, *Salmonella typhimurium* TA 98, TA100, TA1535 and TA1537 and *Escherichia coli* [WP2 (uvrA)] were used with the dose range from 0.0015 to 0.16 µg/plate in triplicates with and without S9 activation. In the chromosomal aberration test with mammalian cells, a Chinese hamster lung fibroblast cell line (CHL/IU) in culture was used at dose levels 0.34, 0.69, 1.37, 2.75 and 5.50 mg/mL in the absence and presence of the metabolic activation. CVA1020 induced no significant increases in the number of revertants in any of the strains at the dose levels where antibacterial effects were not noted with and without metabolic activation. CVA1020 did not induce increase in the incidence of cells with chromosomal aberration or those with genome mutation (polyploidy) in any of the strains irrespective of the absence or presence of metabolic activation. Thus, based on these results it is concluded that CVA1020 does not have mutagenic potential.

Keywords: Antibiotic adjuvant entity, CVA1020, reverse mutation test

INTRODUCTION

Drugs that induce mutation can potentially damage the germ lines leading to fertility problems and to mutations in future generations (Mortelmans and Zeiger, 2000). In recent years, genotoxicity has become an important study in the process of screening for potential development of drugs (Muster *et al.*, 2003). Generally, before setting of clinical trials of a novel drug, its safety must be evaluated by genotoxicity test. The most frequently used methods for genotoxicity include bacterial reverse mutation (Ames) test, chromosome aberration test and micronucleus test which are recommended by regulatory agencies for determining genetic risk (Korea Food and Drug Administration, U.S. Food and Drug Administration, (Organization for Economic Cooperation and Development, 1997; Mortelmans and Zeiger, 2000; Sunder and Green, 2001).

The Ames test is a biological assay that is used worldwide to evaluate the mutagenic activity of new medicines (Maron and Ames, 1983). The chromosome aberration test, which used mammalian cells, is typically measured by observing the cell's chromosome breaking or rearrangements and is proven *in vitro* short-term assay to evaluate the genotoxic risks of drugs (Ishidate and Odashima, 1977). The studies carried out in bacteria and mammalian cells showed that some chemicals interacted with the genetic material and caused DNA damage (Mirian, 2004). The DNA damage

caused by genotoxic agents may become apparent morphologically (Çelik and Eke, 2011).

Vancomycin (a glycopeptide drug) has been used worldwide against Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections. Recently, however, it is losing potency against *S. aureus*, including MRSA (Sakoulas and Moellering, 2008). A recent study showed 76% treatment failure rate with vancomycin (Howden *et al.*, 2004). Similarly, the rate of non-susceptibility of penicillin-resistant *Streptococcus pneumoniae* strains to ceftriaxone is increasing significantly (Chiu *et al.*, 2007; Karunakaran *et al.*, 2012). In view of increasing resistance, Venus Medicine Research Centre, India has developed a new combination product which was named as CVA1020. CVA1020 is a novel antibiotic adjuvant entity comprised of a β -lactam moiety (ceftriaxone) plus a glycopeptide (vancomycin) along with a non antibiotic adjuvant entity L-arginine. Clinically, this antibiotic combination has been designed for the treatment of infections caused by multi drug-resistant gram-positive and gram-negative organisms. As a part of safety test of CVA1020, its genotoxicity was investigated by an Ames test and *in vitro* chromosomal aberration test. Therefore, the aim of the present study was to assess the mutagenic potential of CVA1020 using the bacterial reverse mutation assay (Ames test) with bacteria and *in vitro* chromosomal aberration test with mammalian cells.

MATERIALS AND METHODS

The study was conducted from 15 October, 2012 to 12 November 2012. at Venus Medicine Research Centre, Baddi, Himanchal Pradesh, India.

Drug and chemicals: A novel Antibiotic Adjuvant Entity (AAE), with ceftriaxone sodium plus vancomycin in ratio of 2:1 plus 336 mg arginine herein after referred to as CVA1020 provided by Venus Remedies Limited, Baddi India was dissolved in solvent supplied with the pack. All the chemicals used in the study were of analytical grade. As positive control, sodium azide (NaN₃, Sigma), 9-aminoacridine, (9AA, Aldrich Chemical Company Limited), 2-nitrofluorene (NF, Aldrich Chemical Company Limited), benzo [a] pyrene (BP, Aldrich Chemical Company Limited), 2-aminoanthracene (2-AAN, Aldrich Chemical Company Limited), methyl methansulfonate (MMS, Aldrich Chemical Company Limited) were used. The 2-NF and 9-AA were prepared in sterile distilled water, whereas NaN₃, MMS and BP were prepared in dimethyl sulfoxide (DMSO). Tryptophan and histidine were obtained from Thomas Baker (Chemicals) Limited, Mumbai, India. NADPH was obtained from Himedia (Mumbai, India).

Bacterial strains, cells and S9: The *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2uvrA were purchased from Institute of Microbial Technology, Chandigarh, India. The strain selection complies with the published recommendations (Gatehouse *et al.*, 1994; International Conference on Harmonization, 1995). Chinese Hamster Lung (CHL) cells were obtained from the National Centre for Cell Science (NCCS), Pune, India. A rat liver S9 fraction induced by Aroclor 1254 in male Sprague-Dawley rats was purchased from Trinova Biochem GmbH, Germany. The cells have been kept and passaged at our laboratory using the Eagle's Minimum Essential Medium (MEM, Himedia, Mumbai, India) supplemented with 10% calf serum containing 0.12% sodium bicarbonate.

Bacterial reverse mutation (Ames) test: The mutagenicity test was conducted using the plate incorporation method as described by Ames *et al.* (1975) and Maron and Ames (1983). Briefly, frozen stock cultures of bacterial cells were inoculated into 3.0% of nutrient broth (Himedia, Mumbai, India) and was incubated with gentle shaking at 37±2°C for about 14-16 h until a cell density of about 10⁹ cell/mL was obtained (determined by optical density). The standard mix of S9/cofactor comprised of 2.1 mL (4%) of rat liver S9 (Aroclor-1254 induced), 1.05 mL of salt solution (1.65 M KCl+0.4 M MgCl₂), 0.26 mL of 1 M glucose-6-phosphate, 2.1 mL of 0.1 M NADP solution,

26.25 mL of 0.2 M sodium phosphate buffer, pH 7.4 and 20.74 mL sterile distilled water. The minimal glucose agar plate used for the mutagenicity assay consisted of 1.5% agar supplemented with 2.0% glucose and 2.0% Vogel-Bonner medium E. The top agar consisted of 0.6% agar and 0.5% NaCl, was supplemented right before use with 0.5 mM solution of histidine (for *Salmonella*) and tryptophan (for *E. coli*).

The required quantity of CVA1020 (0.0015, 0.005, 0.016, 0.05 and 0.16 µg/plate) was added into the tube containing 0.5 mL of S9/cofactor mix, 0.1 mL of bacterial suspension (1-2×10⁹ cells/mL) of each strain (TA1535, TA1537, TA98, TA100 and WP2 uvrA) and 2.0 mL top agar. The tubes were vortexed, poured onto minimal glucose plates and evenly distributed. The agar was allowed to harden and the plates were inverted and incubated at 37°C±2°C for 72±4 h, then scored. All experiments were run in triplicates. Similarly, positive and negative controls were also run.

A positive result is defined if either a two fold increase over the spontaneous reversion rate (percent of controls >200%) or demonstration of a dose-response curve when dilutions are tested and can be non-mutagenic if either a less than two-fold increase over spontaneous reversion rate (percent of control <200%) or no dose response curve when dilutions are tested.

Counting procedure and data presentation: All plates for all concentrations were counted by hand. Data are presented as the number of revertant colonies per plate. All data represent the mean±SD of three independent experiments.

In vitro chromosomal aberration test: *In vitro* chromosomal aberration test was performed according to OECD guidelines. To perform *in vitro* chromosomal aberration test, in the direct method without metabolic activation, the cells in 5 mL of cell suspension (5000 cells/mL) were seeded in a 60 mm plastic culture dish and incubated for 24 and 48 h. The solution of CVA1020 (0.312, 0.625, 1.25, 2.50 and 5.0 mg/mL) or mitomycin C (MMC) (0.00005 and 0.0001 mg/mL) was added to the culture cells. The test compound was allowed to remain in the cultures for 24 or 48 h. To arrest cells in metaphase 100 µL of 10 µg/mL colchicine (Sigma) was added to all cultures 2 h before harvest and chromosome preparations were made as described earlier (Dusinska *et al.*, 2003).

For the metabolic activation, 0.5 mL of S9 mix and various concentrations of (0.34, 0.69, 1.37, 2.75 and 5.50 mg/mL) or 0.02 mg/mL of Benzopyrine (BP) was added to 3 day old cultured cells. Cells were treated for 6 hours and washed with Dulbecco's phosphate buffer solution (pH 7.4) and then re-cultured with a new culture medium for 18 h. The chromosomal preparations were made as described earlier (Dusinska *et al.*, 2003). The frequency of the cells with structural

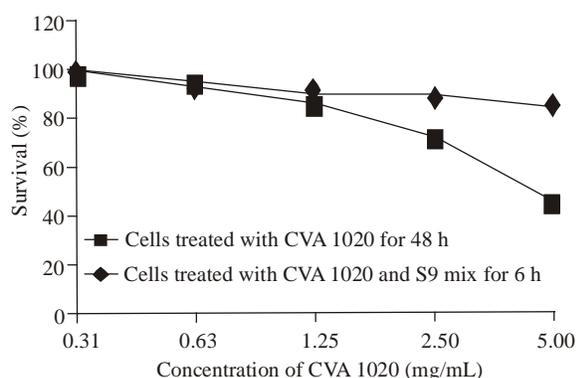


Fig. 1: Survival curves of CHL treated with CVA1020: cell treated with CVA1020 for 48 h and cell treated with CVA1020 and S9 mix for 6 h; survival was expressed as percentage of treated group to solvent control group

and numeric chromosomal aberrations were scored in 100 well spread metaphase for each dose. Types of structural chromosomal aberrations were classified into following groups: chromatid breaks (ctb), chromatid exchange (cte), chromosome breaks (csb), chromatid and chromosomal gap (ctg) and chromosome exchanges (cse) including dicentric and ring chromosomes total cells which have chromosome aberrants including ctg (TAG), total cells which have chromosomal aberrants excluding ctg (TA). The final results were judged as follows: negative (-) if the frequency of aberrant cells was <5%, inconclusive (±) if ≥5 % but <10 % and positive (+) if ≥10 %.

Dosage: Prior to the reverse mutation assay, CVA1020 was evaluated for the cytotoxicity of the indicator strains with and without S9 mix to estimate the dosages to be used. As an antibacterial effect was observed at more than 0.16 µg/plate, hence 0.16 µg/plate was used as maximum concentration and four more diluted concentrations 0.0015, 0.005, 0.016 and 0.05 µg/plate were used.

The chromosome aberration test was performed under the following conditions: a short treatment (6 h) with and without S9 and a continuous treatment for 24 and 48 h to determine the mutagenic potential of CVA1020 using CHL/IU cells, a fibroblast cell line.

The survival ratio of cells treated with the test compound for 24 or 48 h in the direct method or for 6 h following recovery time of 18 h in the metabolic activation method are shown in Fig. 1. The concentration showing 50% inhibition of cell growth was estimated to be around 3.50 mg/mL in the direct method, but could not be obtained in the metabolic activation method because the cell growth was inhibited by only 15 % at the dose of 5.0 mg/mL. Therefore, the testing doses of both methods were decided to be 5.0 mg/mL (the maximum dose), 2.50, 1.25, 0.625 and 0.312 mg/mL with common ratio of 2.

Statistical analysis: Data were analyzed using Graph Pad InStat-3 and expressed as mean±Standard Deviation (SD) of three independent experiments. The continuous variables were tested with one-way analysis of variance (ANOVA) and Dunnett's test values <0.05 was considered statistically significant.

Table 1: Mutagenicity assays for CVA1020 with and without-metabolic activation using *Salmonella typhimurium* and *Escherichia coli* strains

			Average of revertant colonies (mean±SD)				
			Base-pair substitution			Frame shift	
Concentration of test material µg/plate			TA100	TA1535	WP2uvrA	TA98	TA1537
CVA1020	S9 mix (-)	0 ^a	108±3	26±3	73±3	28±2	12±2
		0.0015	107±2	26±2	74±6	27±2	11±1
		0.005	106±5	26±2	73±4	27±4	12±2
		0.016	106±5	26±2	71±5	27±3	10±1
		0.05	106±3	24±3	71±6	26±3	10±2
	S9 mix (+)	0 ^a	106±5	23±2	72±4	26±2	11±2
		0.0015	112±9	28±4	75±4	28±2	13±1
		0.005	111±7	27±3	76±2	29±3	12±3
		0.016	109±9	26±5	75±5	30±2	13±1
		0.05	108±8	27±3	75±6	29±2	13±2
+Control	S9 mix (-)	Compound	NaN3	NaN3	MMS	2-NF	9-AA
		Concentration (µg/plate)	1.5	1.5	2.5	2.5	25
		Colony no	502±9	324±7	397±7	315±4	77±3
		Compound	2-AAN	2-AAN	2-AAN	BP	BP
		Concentration (µg/plate)	10	10	10	20	20
	S9 mix (+)	Compound	519±5	396±8	380±5	315±2	82±2
		Concentration (µg/plate)	10	10	10	20	20
		Colony no	519±5	396±8	380±5	315±2	82±2
		Compound	10-50	60-220	5-50	1-25	65-115
		Concentration (µg/plate)	10	10	10	20	20

Historical negative^b

^a: Negative (solvent) control; ^b: the historical negative range was formed by reference literature; test article can be mutagenic if either a two fold increase over the spontaneous reversion rate (percent of controls >200%) or demonstration of a dose-response curve when dilutions are tested and can be a non-mutagenic if either a less than two-fold increase over spontaneous reversion rate (percent of control <200%) or no dose response curve when dilutions are tested

Table 2: Chromosome aberration test of CVA1020 in CHL cells

Compound	S9	Time (h)	Dose (mg/mL)	Scored cell no	Polyploid (%)	Judge	Frequency of cells with chrosomal aberrations (%)							
							ctg	ctb	cte	csb	cse	TAG	TA	
Solvent	-	24-0 ^a	0	100	0	-	0	0	0	0	0	0	0	
	-	24-0 ^a	0.34	100	0	-	0	0	0	0	0	0	0	
	-	24-0	0.69	100	1	-	1	0	0	0	0	1	0	
	-	24-0	1.37	100	0	-	0	0	0	0	0	1	0	
	-	24-0	2.75	100	0	-	2	1	0	0	0	2	1	
	-	24-0	5.50	100	0	-	2	1	0	0	0	3	0	
MMC	-	24-0	0.00005	100	0	+	42	12	69	0	6	83	75	
	Solvent	-	48-0	0	100	0	-	0	0	0	0	0	0	0
		-	48-0	0.34	100	0	-	0	0	0	0	0	0	0
		-	48-0	0.69	100	0	-	2	0	0	0	0	2	0
		-	48-0	1.37	100	0	-	2	1	0	0	0	3	0
		-	48-0	2.75	100	1	-	1	0	0	0	0	1	0
-		48-0	5.50	100	0	-	1	0	0	0	0	1	0	
MMC	-	48-0	0.0001	100	0	+	29	11	44	0	3	55	47	
	Solvent	-	6-18	0	100	0	-	0	0	0	0	0	0	0
		-	6-18	0.34	100	0	-	0	0	0	0	0	0	0
		-	6-18	0.69	100	0	-	2	1	1	0	0	4	2
		-	6-18	1.37	100	0	-	3	0	0	0	0	3	0
		-	6-18	2.75	100	1	-	2	0	0	0	0	2	0
-		6-18	5.50	100	0	-	1	0	0	0	0	1	0	
BP	-	6-18	0.02	100	0	+	2	0	2	0	0	4	2	
	Solvent	+	6-18	0	100	0	-	1	0	0	0	0	1	0
		+	6-18	0.34	100	0	-	0	0	0	0	0	0	0
		+	6-18	0.69	100	0	-	2	0	0	0	0	2	0
		+	6-18	1.37	100	0	-	0	0	0	0	0	0	0
		+	6-18	2.75	100	0	-	2	1	0	0	0	3	0
+		6-18	5.50	100	0	-	1	1	0	0	0	2	0	
BP	+	6-18	0.02	100	0	-	26	6	44	0	3	58	44	

^a: Treatment time; abbreviation: ctg; chromatid and chromosome gap, ctb chromatid break, cte; chromatid exchange, csb; chrosomal break, cse, chrosomal exchange, TAG; total cells which have chrosomal aberrants including ctg, TA; total cells which have chrosomal aberrants excluding ctg, MMC; mitomycin C, BP, benzopyrine

RESULTS

Ames test: The mutagenic activity of CVA1020 was tested by *in vitro* with and without metabolic activation in *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA and results are presented in Table 1. An increased of revertant colonies were not observed as compared with those of the corresponding solvent controls with or without S9 mix in the tested dose range for any strains. On the other hand, the number of revertant colonies in positive controls increased remarkably.

In vitro chromosomal aberration test: Results of chromosomal aberrations test are shown in Table 2. The incidence of cells having aberrants (including gap) in chromosomal structure was 0-1% and 0-4% in solvent groups and treated groups, respectively and the incidence of aberrant cells excluding gap was 0% and 0-2%, respectively. There were no significant differences were observed in the chromosomal aberration between the treated groups and the corresponding solvent groups. In contrast, the incidence of aberrant cells in each positive control group increased greatly as compared with each solvent group. The incidence of cells having numeral aberrations (polyploid) did not increase in any groups.

DISCUSSION

Bacterial mutagenicity (Ames) test is a biological assay which has been used widely for the initial

screening of mutagenic potential of medical compounds (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). However, this test cannot detect aberrations of chromosome induced by chemicals (Ohuchida and Yoshida, 1988); chromosomal aberrations can be detected only in mammalian cell. Therefore, it has been recommended to use these two tests as a minimum requirement for mutagenicity tests *in vitro* (Ishidate, 1988). The chromosomal aberrations method involves culturing cells, exposing them to a test material, harvesting the cells, preparing cells for microscope analysis and then counting the frequency of abnormal structures and chromosome aberrations (Ishidate and Odashima, 1977). Generally, beta-lactam is considered to be non-mutagenicity. However, mitomycin C and tetracyclin have been demonstrated to mutagenic (Kada and Ishidate, 1980; Celik and Eke, 2011).

In this study, the reverse mutation test of CVA1020 was studied by means of the Ames test and the chromosomal aberration test. According to the data obtained in the present investigation, CVA1020 is non-mutagenic as there was either a less than two-fold increase over spontaneous reversion rate (percent of control <200%) or no dose response curve when dilutions are tested. Moreover, there were no significant differences between results of treated groups in the absence and presence of the metabolic activation for both tests. Similarly, Ohuchida and Yoshida, 1988 studied the mutagenicity of cefodizime sodium on *Salmonella typhimurium* and *Echerichia coli* strains and have observed no mutagenic activity of cefodizime sodium. Cefotaxime (Mazza *et al.*, 1980) and MT-141

(Hirano *et al.*, 1984), which is one of cephamycins were also reported to be non mutagenicity. Contrary to this, several β -lactam antibiotics such as penicillin G, ampicillin and carbenicillin have been reported to be mutagenic on the chosomal aberration test with human's fibroblast cells (Byarugaba *et al.*, 1975); however, based on the reverse mutation test with *S. typhmuri*um, penicillin G found to be non-mutagenic (McCann *et al.*, 1975).

CONCLUSION

In conclusion, the results of this investigation revealed that CVA1020 is non-mutagenic as there was no increased in the number of revertant colonies when compared with those of the corresponding solvent controls with or without S9 mix in the tested dose range for any strains. Similarly, there were no significant differences in the chromosomal aberration between the treated groups and the corresponding solvent groups.

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