DATA EVALUATION RECORD

R182281 (METABOLITE OF CHLOROTHALONIL)

Study Type: OCSPP No Guideline; *In vivo* Comet Test in Rats

EPA Contract No. EP-W-16-018
Task Assignment No. 35-23-018 (MRID 51485515)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
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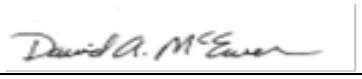
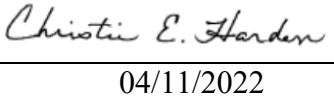
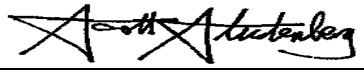
Primary Reviewer: David A. McEwen, B.S.	Signature: Date:	 02/22/2022
Secondary Reviewer: Christie E. Harden, B.S.	Signature: Date:	 04/11/2022
Quality Assurance: Scott D. Studenberg, Ph.D., DABT	Signature: Date:	 04/22/2022
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Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CDM/CSS-Dynamac Joint Venture personnel. Contractor's role did not include establishing Agency policy.

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DATA EVALUATION RECORD

STUDY TYPE: *In Vivo* Comet Test in Rats; OCSPP No Guideline; OECD 489.

PC CODE: 081901 **DP BARCODE:** D468198
TXR #: 0058619

TEST MATERIAL (PURITY): R182281 (metabolite of chlorothalonil; 98.7% a.i.)

SYNONYMS: 2,4,5-trichloro-6-hydroxy-1,3-benzenedicarbonitrile

CITATION: Herring, T. (2019) R182281 - Crl:CD(SD) rat *in vivo* Comet test. Covance CRS Limited, Alconbury, Huntingdon, Cambridgeshire, UK. Laboratory Study No.: NR52CX, August 14, 2019. MRID 51485515. Unpublished.

SPONSORS: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, UK

Sipcam Oxon S.p.A., Via Sempione 195, Pero, Milan, Italy

SCIENTIFIC INTEGRITY: The conclusions conveyed in this assessment were developed in full compliance with *EPA Scientific Integrity Policy for Transparent and Objective Science*, and EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/sites/default/files/2014-02/documents/scientific_integrity_policy_2012.pdf. The full text of the EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

EXECUTIVE SUMMARY: In a non-guideline, *in vivo* Comet test (MRID 51485515), groups of six male Crl:CD(SD) rats/dose level were administered R182281 (a metabolite of chlorothalonil; 98.7% a.i.; Batch No. P2;) in 0.5% methylcellulose (dose volume 20 mL/kg) via oral gavage at dose levels of 0, 31.25, 62.5, or 125 mg/kg/day. The rats were administered the test compound twice approximately 24 hours apart, and euthanized approximately 4 hours after administration of the second dose. A positive control group of three male Crl:CD(SD) rats was administered a single dose of ethyl methanesulfonate in purified water at a dose level of 200 mg/kg (dose volume 10 mL/kg) and euthanized approximately 3 hours post-dose. Samples of liver and duodenum were removed from each rat; single-cell suspensions were prepared for each sample and used to prepare Comet slides. The extent of DNA migration was quantitated as % tail intensity (%TI).

Prior to the Comet test, a preliminary toxicity test was performed to establish the maximum tolerated dose (MTD): groups of two Crl:CD(SD) rats/sex/dose level were administered R182281 in 0.5% carboxymethylcellulose (dose volume 20 mL/kg) via oral gavage at dose levels of 125 or 250 mg/kg/day. The rats were administered the test compound twice approximately 24 hours apart, and monitored for excessive toxicity (mortality, clinical signs of toxicity, and decreased body weights) following the first dose and up to 2-6 hours after the second dose. These rats were then euthanized and discarded. Additionally, a satellite group of three male Crl:CD(SD) rats were treated with a single dose of the test compound as above at a dose level of 125 mg/kg and were used to establish a time of maximum concentration (T_{max}) in plasma.

Preliminary toxicity study: The MTD was established at 125 mg/kg/day. Male and female rats administered 250 mg/kg/day displayed hunched and flattened posture, elevated gait, increased touch response, slow breathing, piloerection, whole body twitches, reduced body tone, decreased activity, grinding teeth, and convulsions; all rats were euthanized for humane reasons following dosing on Day 2. At 125 mg/kg/day, no clinical signs were observed in the males and in the females clinical signs were limited to hunched posture, elevated gait, irregular breathing, and piloerection in one rat each on Day 2. Body weights were slightly decreased (<5%) in the 125 and 250 mg/kg/day males and females from Day 1 to Day 2.

Comet test: All rats survived to scheduled euthanasia and no clinical signs of toxicity were observed at any dose including the vehicle and positive controls. Body weights were slightly decreased (<3%) in the ≥62.5 mg/kg/day rats from Day 1 to Day 2.

There were no treatment-related increases in either mean or median %TI in the livers or duodenum of male rats treated with R182281. At 125 mg/kg/day, the group mean of median %TI in the liver was increased; however, the individual and group (mean and median) %TI values from all treatment groups were within the laboratory provided vehicle historical control ranges. Therefore, this finding was not considered to be of biological relevance and R182281 was considered to give a negative result in this assay.

Treatment with the positive control (200 mg/kg ethyl methanesulfonate) caused a 14-fold increase in mean %TI and 99-fold increases in median %TI in liver, and a 8.3-fold increase in mean %TI and 18-fold increase in median %TI in duodenum. There were no significant increases in the number of Hedgehog cells in any group.

Plasma analysis: The time of maximum mean plasma concentration (T_{max}) for a single 125 mg/kg dose of R182281 was determined to be 4-8 hours post-dosing (plasma concentrations were comparable at 4 and 8 hours post-dose).

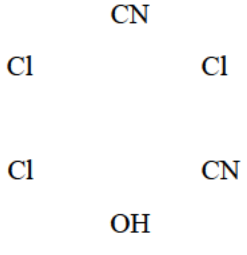
This study is classified **Acceptable/Non-guideline** and demonstrates that R182281 is non-mutagenic as determined by the Comet test under the conditions of the present study.

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: R182281 (a metabolite of chlorothalonil)

Description:	White solid
Batch No.:	P2
Purity:	98.7% (w/w) a.i.
Expiration/Storage:	November 30, 2022/Refrigerated (2-8°C)
CASRN (parent):	28343-61-5 (1897-45-6)
Structure:	
Positive control	Ethyl methanesulfonate
Batch No:	BCBW8635 (Sigma)

2. Vehicle: 0.5% carboxymethylcellulose

3. Test animals:

Species:	Rat
Strain:	Crl:CD(SD)
Age/weight at study initiation:	Preliminary test: 48-61 days / males 225-279 g; females 197-212 g Bioanalysis phase (males ony): 48-54 days / 211-217 g Comet test (males only): 48-54 days / 211-243 g
Source:	Charles River UK Limited (Margate, Kent, England)
Housing:	Group-housed (2-3) by sex in cages (details regarding cages not provided).
Diet:	Pelleted Envigo Teklad 2014C, *ad libitum*
Water:	Tap water, *ad libitum*
Environmental conditions:	**Temperature:** 20-24°C **Humidity:** 40-70% **Air changes:** Not reported **Photoperiod:** 12 hours light/12 hours dark
Acclimation period:	At least 5 days

B. STUDY DESIGN

1. Experimental dates: Start: March 15, 2019 End: June 25, 2019

2. Animal assignment: Animals were randomly assigned (method not reported) to the test groups presented in Tables 1a and 1b.

TABLE 1a. Preliminary toxicity test [a]

Group #	Concentration (mg/mL)	Dose (mg/kg/day)	# of rats	
			Male	Female
1	12.5	250	2	2
2	6.25	125	2	2
3 [b]	6.25	125	3	0

a Data were obtained from page 18 of MRID 51485515.
b Satellite rats (bioanalysis phase) for plasma to identify T_{max}.

TABLE 1b. Comet test [a]			
Group	Concentration (mg/mL)	Dose (mg/kg/day)	# of rats Male
Control	0.0	0	6
Low	1.56	31.25	6
Mid	3.13	62.5	6
High	6.25	125	6
Positive control [b]	20	200	3

a Data were obtained from page 19 of MRID 51485515.
b Ethyl methanesulfonate administered once.

3. **Dose selection rationale:** A dose selection rationale was not provided. It was stated that the preliminary toxicity test was performed to identify the maximum tolerated dose (MTD) of R182281. Based on the results of the preliminary toxicity test, the MTD was exceeded at 250 mg/kg/day but was established at 125 mg/kg/day with no clinical signs of toxicity in males and limited clinical signs in females, and no gross pathology findings after administration for two days. Body weights were slightly decreased (<5%) in both the 125 and 250 mg/kg/day males and females from Day 1 to Day 2. Satellite rats were administered two doses of R182281 at 125 mg/kg/day to establish the time of maximum concentration (T_{max}) in plasma. For the definitive Comet test, dose levels of 31.25, 62.5, and 125 mg/kg/day were administered. Ethyl methanesulfonate, a known mutagen, was administered as a single dose of 200 mg/kg to the positive control group.

4. **Test formulations and analyses:** The appropriate amount of the test compound was weighed and ground in a mortar with a pestle; 90% of the required volume of vehicle was gradually added during continuous rubbing. The pH was checked and if below pH 3.0, the formulation was adjusted to pH 3.0-5.0 with 10 M sodium hydroxide and brought up to volume with remaining vehicle. The pH was considered acceptable for oral dosing. Each concentration was prepared separately, stirred, and the pH recorded. The positive control was dissolved in purified water to yield a concentration of 20 mg/mL.

   Stability of the test material in 0.5% carboxymethylcellulose was assessed at 1 and 100 mg/mL after room temperature storage (15-25°C) for 0, 1, and 2 hours (with continuous stirring) and after one day and after storage at 2-8°C for 1 and 8 days. Homogeneity and concentration analyses were performed on duplicate samples from the top, middle, and bottom of the test suspensions; it was assumed that the formulations used in the preliminary toxicity test were not analyzed.

Results

Homogeneity (% CV): 0.14-2.64%

Stability (% of time 0): 99.0-100.8% (up to 24 hours at room temperature)
100.0-101.6% (up to 8 days refrigerated)

Concentration (% of nominal): 98.6-100.3%

The analytical data indicated that the mixing procedure was adequate and that the variance between nominal and actual dosage to the animals was acceptable.

5. **Test substance administration:** For the preliminary toxicity test, the rats were administered the test compound twice (dose volume 20 mL/kg) approximately 24 hours apart, and monitored for excessive toxicity (mortality, clinical signs of toxicity, and decreased body weights) after the first dose and up to 24 hours after the second dose. For the Comet test, the rats were administered the test compound twice (dose volume 20 mL/kg) approximately 24 hours apart, and euthanized approximately 4 hours after administration of the second dose. A positive control group of three male Crl:CD(SD) rats were administered a single dose of ethyl methanesulfonate in purified water at a dose level of 200 mg/kg (dose volume 10 mL/kg) and euthanized approximately 3 hours later.

6. **Rationale for use of the Comet test:** The Single Cell Gel Electrophoresis (SCGE) assay, or Comet assay, is a rapid, visual, and quantitative technique for measuring DNA strand breakage in individual mammalian cells. The comet assay (Singh *et al.*, 1988[1]) uses a microgel technique involving electrophoresis under alkaline (pH >13) conditions for detecting DNA damage, in the form of single-strand breaks and alkali-labile sites, in virtually any eukaryotic cell population that can be obtained as a single-cell suspension. Cell suspensions from any tissue taken from animals treated *in vivo* are embedded in agarose gel on glass microscope slides and lysed by detergent and high salt solution to rupture the cell membranes, extract the nuclear proteins, and leave the supercoiled DNA in a nucleus-type structure called the nucleoid. Subsequently, the supercoiled DNA is left to relax and 'unwind' in a strong alkaline buffer. As DNA carries a net negative charge, the non-supercoiled loops and single-strand fragments migrate toward the anode during electrophoresis. If DNA strand breaks occur during the chemical insult, the open loops or fragments migrate further within the gel. After electrophoresis, the DNA is neutralized and stained with a nucleic acid-specific dye. The cells are visualized and quantitated by using fluorescence microscopy linked to an image analysis system.

7. **Statistics:** For the Comet test, group means of median tail intensity (%TI) were analyzed statistically; significance was noted at $p<0.05$.

   For comparison of the vehicle control and R182281-treated groups, Bartlett's test for homogeneity of variances was performed. If Bartlett's test was not significant ($p>0.01$), parametric analysis using the F1 approximate test was performed. If the F1 test was not significant ($p>0.01$), Williams' test for a monotonic trend was performed; otherwise, Dunnett's test was performed. If Bartlett's test was significant ($p<0.01$), logarithmic and square-root transformations were applied (if a parameter contained any zero values, 0.001 was added to all values for that parameter prior to logarithmic transformation). If Bartlett's test was still significant ($p<0.01$), non-parametric analysis with the H1 approximate test was performed. If the H1 test was not significant ($p>0.01$), Shirley's test for a monotonic trend was performed; otherwise, Steel's test was performed.

[1] Singh, N.P., McCoy, M.T., Tice, R.R. *et al*. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research, **175:** 184-191.

For comparison of the vehicle control and positive control groups, Bartlett's test was performed. If Bartlett's test was not significant ($p>0.01$), parametric analysis with a t-test was performed. If Bartlett's test was significant ($p<0.01$), logarithmic and square-root transformations were applied as above. If Bartlett's test was still significant ($p<0.01$), non-parametric analysis with the Wilcoxon rank sum test was performed.

The statistical methods were considered appropriate.

8. **Historical control data:** Historical control data for liver and duodenum vehicle control and positive control mean %TI and median %TI values were presented in Appendix 3 on pages 47-48 of MRID 51485515. The included studies were conducted during March 2017 through March 2019 and consisted of 106 individual rats in 19 studies for vehicle control and 58 individual rats in 18 studies for positive control for liver, and 78 individual rats in 14 studies for vehicle control and 42 individual rats in 14 studies for positive control for duodenum.

C. METHODS

1. **Clinical observations:** Any rats observed with severe signs were euthanized for humane reasons. For the preliminary toxicity test, the rats were observed at regular intervals during the work day. For the Comet test, it was reported that the rats were observed regularly for evidence of mortality and clincal signs of toxicity throughout the work day.

2. **Body weight:** All rats were weighed on the day following arrival, on each day of dosing, and prior to euthanasia.

3. **Euthanasia and tissue sampling:** Preliminary toxicity test rats were euthanized (method not described) approximately 24 hours following the second dose and discarded. Comet test rats were euthanized (method not described) 4 hours after the second dose. Samples of liver and duodenum were excised and put into ice-cold mincing solution (not described). Single-cell suspensions were prepared from all samples by using tissue-specific methods for liver and duodenim, respectively.

4. **Comet test:** First, glass slides were dipped in 1% normal melting point agarose and air dried. For each tissue, appropriate dilutions of the single-cell suspensions were prepared and mixed with an appropriate volume of 0.5% low melting point agarose. A 75-µL portion of each mixture was applied to the coated slides and a cover slip was applied. After the agar had cooled and set, the cover slips were removed and the slides were placed in a light-proof box containing chilled lysis buffer and stored at 2-8°C overnight. On the following day, the slides were randomly placed on a dry, level platform in a horizontal electrophoresis unit containing chilled electrophoresis buffer. The unit was filled with electrophoresis buffer until the tops of the slides were covered and maintained at 5-9°C. The slides were incubated for 20 minutes to allow for DNA unwinding. After incubation, electrophoresis was performed at 18 V with a starting current of approximately 300 mA for 30 minutes. After electrophoresis, the slides were washed 3 × 5 minutes with neutralization buffer and stored in refrigerated, light-proof boxes containing moistened tissues as a humidity source. The

slides were coded, treated with a fluorescent nucleic acid stain (SYBR™ Gold), and examined with a fluorescence microscope by using a CCD camera and a commercial image analysis system (COMET IV™). The slides were first analyzed for overt toxicity, defined as an increase in background debris and/or an increase in the incidence of excessively damaged cells (hedgehog cells). These cells and cells with staining artefacts were excluded from analysis. Fifty cells/slide were scored (150 cells/tissue/rat) and the extent of DNA migration (damage) was quantitated as %TI (*i.e.*, the fluorescence detected by the image analysis in the tail which was proportional to the amount of DNA that had moved from the head region to the comet tail).

5. **Plasma analysis:** Blood samples (0.3 mL) were obtained from the satellite rats of the preliminary toxicity test (three males administered 125 mg/kg/day R182281) at 0.5, 1, 2, 4, 8, and 24 hours post-dosing; samples were collected into K_2EDTA and centrifuged to harvest plasma. Plasma samples were analyzed by LC-MS/MS to determine T_{max}.

II. RESULTS

A. PRELIMINARY TOXICITY TEST

1. **Mortality:** All male and female rats administered 250 mg/kg/day were euthanized for humane reasons after the second dose. All male and female rats administered 125 mg/kg/day survived to scheduled euthanasia.

2. **Clinical signs of toxicity:** Clinical signs are presented in Table 2. At 125 mg/kg/day, no clinical signs were observed in the males and in the females clinical signs were limited to hunched posture, elevated gait, irregular breathing, and piloerection in one rat each on Day 2. Male and female rats administered 250 mg/kg/day displayed hunched and flattened posture, elevated gait, increased touch response, slow breathing, piloerection, whole body twitches, reduced body tone, decreased activity, grinding teeth, and convulsions; all rats were euthanized for humane reasons following dosing on Day 2. The MTD was established at 125 mg/kg/day.

TABLE 2. Preliminary toxicity test clinical signs (observations Day 1/Day 2). [a]

Observation	Dose (mg/kg/day)			
	125		250 [b]	
	Males	Females	Males	Females
Hunched posture	0/0	0/1	0/2	0/2
Flattened posture	0/0	0/0	0/1	0/1
Elevated gait	0/0	0/1	0/1	0/1
Increased touch response	0/0	0/0	0/2	0/2
Slow breathing	0/0	0/0	0/2	0/2
Irregular breathing	0/0	0/1	0/0	0/0
Piloerection	0/0	0/1	0/1	0/1
Whole body twitches	0/0	0/0	0/1	0/1
Reduced body tone	0/0	0/0	0/1	0/1
Decreased activity	0/0	0/0	0/1	0/1
Grinding teeth	0/0	0/0	0/0	0/1
Convulsion	0/0	0/0	0/0	0/1

a Data obtained from Appendix 2 on pages 44-45 of MRID 51485515; n = 2/sex.
b All rats euthanized for humane reasons after the second dose.

3. **Body weight:** Body weight data are presented in Table 3 for completeness only. Due to the short duration of the test (<48 hours), little information on systemic toxicity can be obtained from the body weights. Body weights were slightly decreased (<5%) in the 125 and 250 mg/kg/day males and females from Day 1 to Day 2.

TABLE 3. Mean (± SD) body weights (g) in Crl:CD(SD) rats administered two doses of R182281 via oral gavage. [a]

Study Day	Dose (mg/kg/day)	
	125	250 [b]
Males		
Day 1	279 ± 0.7	230 ± 6.4
Day 2	269 ± 0.0	221 ± 7.8
Day 3 (at euthanasia)	271 ± 4.9	--
Females		
Day 1	210 ± 2.8	197 ± 0.0
Day 2	201 ± 0.0	193 ± 1.4
Day 3 (at euthanasia)	200 ± 4.9	--

a Data were obtained from Appendix 1 on page 42 of MRID 51485515; n = 2/sex.
b All rats euthanized for humane reasons following the second dose.

B. COMET TEST

1. **Mortality:** All rats survived to scheduled euthanasia.

2. **Clinical signs of toxicity:** No clinical signs of toxicity were noted at any dose including the vehicle and positive controls.

3. **Body weight:** Body weight data are presented in Table 4 for completeness only. Due to the short duration of the test (<48 hours), little information on systemic toxicity could be obtained from the body weights. Body weights were slightly decreased (<3%) in the ≥62.5 mg/kg/day rats from Day 1 to Day 2.

TABLE 4. Mean (± SD) body weights (g) in male Crl:CD(SD) rats administered two doses of R182281 via oral gavage. [a]

Study day	Dose (mg/kg/day)				
	0	31.25	62.5	125	Positive control [b]
Day 1	226 ± 11.8	229 ± 5.0	228 ± 11.7	227 ± 6.9	--
Day 2	233 ± 10.0	236 ± 4.5	222 ± 15.1	222 ± 5.5	232 ± 4.2
At euthanasia	227 ± 10.4	233 ± 5.2	219 ± 13.9	218 ± 10.5	230 ± 3.1

a Data were obtained from Appendix 1 on page 43 of MRID 51485515; n = 6 rats/dose group for R182281; n = 3 rats for positive control.
b 200 mg/kg ethyl methanesulfonate.

4. **Comet test:** Comet test data are presented in Table 5. There were no treatment-related increases in either mean or median %TI in the livers or duodenum of male rats treated with R182281. At 125 mg/kg/day, the group mean of median %TI in the liver was increased ($p<0.05$); however, the individual and group (mean and median) %TI values from all treatment groups were within the laboratory-provided vehicle historical control ranges. Therefore, this finding was not considered to be of biological relevance and R182281 was considered to yield a negative result in this assay.

Treatment with the positive control (200 mg/kg ethyl methanesulfonate) caused a 14-fold increase in mean %TI and 99-fold increase ($p<0.001$) in median %TI in liver and a 8.3-fold increase in mean %TI and 18-fold increase ($p<0.001$) in median %TI in duodenum.

Although there were slight dose-dependent increases at all doses in the number of Hedgehog cells noted in the liver (0.8, 1.3, and 2.5 at 31.25, 62.5, and 125 mg/kg/day, respectively vs. 0 control) and duodenum (4.2, 10.3, and 12.7 at 31.25, 62.5, and 125 mg/kg/day, respectively vs. 1.7 control), none of the values achieved statistical significance and are therefore not of concern.

TABLE 5. Group means (± SD) of mean and median tail intensity (%TI) in male Crl:CD(SD) rats administered two doses of R182281 via oral gavage. [a]

Parameter	Dose (mg/kg/day)						
	0	Historical control [c]	31.25	62.5	125	Positive control[b]	Historical control[b]
Liver							
# cells scored	900		900	900	900	450	
%TI	1.98 ± 0.5	1.5-3.8	1.85 ± 0.4	1.92 ± 0.3	2.14 ± 0.4	27.88 ± 1.6	20.5-53.9
Median %TI	0.26 ± 0.2	0.1-0.9	0.22 ± 0.1	0.28 ± 0.2	0.48 ± 0.2*	25.73 ± 0.9***	18.2-54.9
Duodenum							
# cells scored	900		900	900	900	450	
%TI	4.47 ± 0.6	2.8-6.7	4.17 ± 0.4	4.31 ± 0.6	4.22 ± 0.7	37.28 ± 5.9	29.4-50.3
Median %TI	1.98 ± 0.6	0.3-3.0	1.71 ± 0.5	1.80 ± 0.2	1.50 ± 0.8	34.97 ± 7.0***	26.0-50.5

a Data were obtained from Table 1 on page 33 and from Appendix 3 on pages 47-48 of MRID 51485515; n = 6 rats/dose group for R182281; n = 3 rats for positive control.
b 200 mg/kg ethyl methanesulfonate.
c Includes data for Wistar Han and Sprague-Dawley rats.
* Signifcantly different from control; $p<0.05$.
*** Significantly different from control; $p<0.001$.

C. PLASMA ANALYSIS: Plasma concentration data were presented in Appendix 5 of the MRID and are included as Appendices I and II at the end of the DER. The time of maximum mean plasma concentration (T_{max}) for a single 125 mg/kg dose of R182281 was determined to be 4-8 hours post-dosing (plasma concentrations were comparable at 4 and 8 hours post-dose).

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATORS' CONCLUSIONS: R182281 did not show evidence of causing an increase in DNA strand breaks in the liver and duodenum of male Crl:CD(SD) rats when administered orally by gavage in this *in vivo* test system and was negative in this *in vivo* comet assay.

B. REVIEWER COMMENTS: The Reviewers agree with the Investigators' Conclusions.

Preliminary toxicity study: The MTD was established at 125 mg/kg/day. Male and female rats administered 250 mg/kg/day displayed hunched and flattened posture, elevated gait, increased touch response, slow breathing, piloerection, whole body twitches, reduced body tone, decreased activity, grinding teeth, and convulsions; all rats were euthanized for

humane reasons after dosing on Day 2. At 125 mg/kg/day, no clinical signs were observed in the males; in the females, clinical signs were limited to hunched posture, elevated gait, irregular breathing, and piloerection in one rat each on Day 2. Body weights were slightly decreased (<5%) in the 125 and 250 mg/kg/day males and females from Day 1 to Day 2.

Comet test: All rats survived to scheduled euthanasia and no clinical signs of toxicity were observed at any dose including the vehicle and positive controls. Body weights were slightly decreased (<3%) in the ≥62.5 mg/kg/day rats from Day 1 to Day 2.

There were no treatment-related increases in either mean or median %TI in the livers or duodenum of male rats treated with R182281. At 125 mg/kg/day, the group mean of median %TI in the liver was increased ($p<0.05$); however, the individual and group (mean and median) %TI values from all treatment groups were within the laboratory-provided vehicle historical control ranges. Therefore, this finding was not considered to be of biological relevance and R182281 was considered to give a negative result in this assay.

Treatment with the positive control (200 mg/kg ethyl methanesulfonate) caused a 14-fold increase in mean %TI and 99-fold increase ($p<0.001$) in median %TI in liver, and a 8.3-fold increase in mean %TI and 18-fold increase ($p<0.001$) in median %TI in duodenum. There were no statistically significant increases in the number of Hedgehog cells in any group.

Plasma analysis: The time of maximum mean plasma concentration (T_{max}) for a single 125 mg/kg dose of R182281 was determined to be 4-8 hours post-dosing (plasma concentrations were comparable at 4 and 8 hours post-dose).

This study is classified **Acceptable/Non-guideline** and demonstrates that R182281 is non-mutagenic as determined by the Comet test under the conditions of the present study.

C. <u>STUDY DEFICIENCIES</u>: None

APPENDIX I Plasma concentrations of R182281 on Day 1 after the second oral administration of R182281 at 125 mg/kg.

Time (hours)	Concentration (mg/L) Animal numbers		
	13	14	15
0.5	275	223	231
1	309	243	271
2	286	251	239
4	309	284	277
8	281	300	299
24	285	126	188

(copied from Appendix 5, page 101 of MRID 51485515)

APPENDIX II Plasma concentrations of R182281 on Day 1 after the second oral administration of R182281 at 125 mg/kg (repeat analyses).

Subject	Time (hours)	Original Conc. (mg/L)	Original Run Number	Reason for Reassay	Reassay Conc. (mg/L)	Reassay Run Number	Reported Conc. (mg/L)	Reason for Reported Conc.
013	0.5	ALQ	1	1	ALQ, 275	2, 3	275	1
013	1	ALQ	1	1	ALQ, 309	2, 3	309	1
013	2	ALQ	1	1	ALQ, 286	2, 3	286	1
013	4	ALQ	1	1	ALQ, 309	2, 3	309	1
013	8	ALQ	1	1	ALQ, 281	2, 3	281	1
013	24	ALQ	1	1	ALQ, 285	2, 3	285	1
014	0.5	ALQ	1	1	ALQ, 223	2, 3	223	1
014	1	ALQ	1	1	ALQ, 243	2, 3	243	1
014	2	ALQ	1	1	ALQ, 251	2, 3	251	1
014	4	ALQ	1	1	ALQ, 284	2, 3	284	1
014	8	ALQ	1	1	ALQ, 300	2, 3	300	1
014	24	ALQ	1	1	ALQ, 126	2, 3	126	1
015	0.5	ALQ	1	1	ALQ, 231	2, 3	231	1
015	1	ALQ	1	1	ALQ, 271	2, 3	271	1
015	2	ALQ	1	1	ALQ, 239	2, 3	239	1
015	4	ALQ	1	1	ALQ, 277	2, 3	277	1
015	8	ALQ	1	1	ALQ, 299	2, 3	299	1
015	24	ALQ	1	1	ALQ, 188	2, 3	188	1

ALQ – Above limit of quantification 1.50 mg/L

Reasons For Reassay:

1). Above limit of quantification (ULOQ), over range

Reasons For Reported Conc:

1). Reassayed result after 600-fold post-extract dilution

(copied from Appendix 5, page 102 of MRID 51485515)