REPORT

TEST 931564

94-03-28 ro

Sponsor	Feinchemie Schwebda GmbH, [redacted] Eupener Str. 150, D-50933 Köln
Date of order	93-12-22
Test	DNA repair test with primary rat hepatocytes (SOP 9.20, OECD Guideline 482)
Test material	GLYPHOSATE, lot no. F/93/032
Arrival of material	93-12-29
Beginning of study	94-02-01
End of study	94-03-18
Study director	[redacted]
Ouality assurance	[redacted]
GLP statement	This study was conducted according to the principles of Good Laboratory Practice.
Data storage	All raw data of this study and a copy of this report are stored in the archives of the supplier.

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Contents

1. Summary

GLYPHOSATE was investigated for its potential to induce genotoxic effects by means of the DNA repair test. Induction of DNA repair synthesis was determined in rat hepatocyte primary cultures by means of the bromodeoxyuridine density-shift method. This technique clearly discriminates DNA repair synthesis from normal replicative DNA synthesis. Repair is identified as incorporation of radioactivity into unreplicated DNA of cells that were incubated in the presence of a radioactive DNA label (^{3}H-deoxycytidine) and a density label (5-bromodeoxyuridine). Repaired DNA is separated from replicated, heavily bromodeoxyuridine-substituted DNA by ultracentrifugation in cesium salt density gradients.

GLYPHOSATE was dissolved in cell culture medium and further diluted in cell culture medium. GLYPHOSATE was tested up to cytotoxic concentrations, and maximal solubility, respectively. With none of the tested extract concentrations a reproducible and significant dose-related increase in radiolabel incorporation was obtained. Therefore, under conditions described GLYPHOSATE is not genotoxic in primary rat hepatocytes.

2. Materials

2.1 Test material

The test compound was provided by Feinchemie Schwebda GmbH, Köln, and was specified as follows: GLYPHOSATE, technical grade, lot F/93/032, > 98 % purity (Report Dr. [redacted] 93-11-05). The compound was stored light-protected at 20 - 25 °C.

2.2 Positive controls

Dimethylnitrosamine (DMN, [redacted] lot 5217133, 98 % purity) and 2-acetamidofluorene (2-AAF, Sigma, München, lot 45F-3657, 96 % purity) served as positive control chemicals. The genotoxicity of both compounds is dependent on metabolic activation by microsomal monooxygenases.

2.3 Tester cells

For the preparation of hepatocytes, adult male Sprague Dawley rats (200 - 350 g body weight, standard strain, GSF-Neuherberg) were used. The animals had received an Altromin standard diet and tap water ad libitum.

2.4 Media and chemicals

Bromodeoxyuridine (BrdUrd), and fluorodeoxyuridine (FdUrd) were obtained from Sigma, München, William's medium E (WME) and fetal calf serum (FCS) from Gibco, Eggenstein, collagenase from Serva (Hep plus 04043), proteinase K from Boehringer, Mannheim; ^{3}H-deoxycytidine (^{3}H-dCyd) from Du Pont de Nemours, Bad Homburg; sodium dodecylsarcosine and Cs_2SO_4 (analytical grade) from Serva, Heidelberg; CsCl (analytical grade) from Fluka, Buchs (Switzerland).

3. Methods

3.1 Test performance

The rat liver was perfused with Ca^{2+}-free modified Hanks' solution containing 100 μM EGTA continued by the same Ca^{2+}-free solution without EGTA. Finally, the liver was perfused with Dulbecco's modified Eagle's Medium (DMEM) containing Ca^{2+} and 0.13 U/ml collagenase. After filtration through a nylon mesh gauze the cells were washed three times in DMEM. Viability of the hepatocyte preparations was greater than 80 %, as determined by exclusion of 0.4 % trypan blue [3].

6 x 10^6 freshly prepared cells were seeded in 75 cm^2 flasks containing 16 ml WME supplemented with 10 % FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml) and allowed to attach for 2 h. The medium was then removed, attached cells were washed twice with 5 ml prewarmed salt solution and incubated for one hour in 4 ml WME containing 40 μM FdUrd and 200 μM BrdUrd.

After preincubation the medium was changed and the incubation continued for 18 h in WME in the presence of 40 μM FdUrd and 200 μM BrdUrd, the test compound (0.07 mM - 48.98 mM (test 1), 0.13 - 111.69 mM (test 2)) and 10 μCi ^{3}H-dCyd/ml (26,7 Ci/mmol). The test compound was dissolved and was serially diluted in WME on day of experiment. The concentration range was determined by pretesting of solubility and of toxicity of the test compound. Controls and reference substances were used as follows: WME (negative solvent control), 10 mM DMN and 0.2 mM AAF. After 18 h incubation cells were washed with phosphate-buffered saline, lysed with sodium dodecylsarcosine and digested with proteinase K. The DNA was precipitated with ethanol, dried, and stored at -80 °C.

The DNA pellets were dissolved and centrifuged to equilibrium in alkaline $CsCl/Cs_2SO_4$ gradients (Beckman rotor VTi 80, 55,200 rpm, 20 °C, 16 h). Following centrifugation, the lower half of each gradient was removed. The remaining part, containing parental DNA strands, was brought to 5 ml with $CsCl/Cs_2SO_4$ and rebanded at 53,000 rpm. After fractionation of the gradients, the UV absorbance and the acid precipitable radioactivity were determined for each fraction [1,2,4].

3.5 Evaluation

Repair synthesis (cpm/μg DNA) was estimated from the gradient profiles by calculating the radioactivity incorporated into light (parental) DNA, i. e. by integrating the radioactivity binding exactly coincident with the UV-absorbance peak, and dividing it by the amount of parental DNA. DNA was quantitated by integrating the UV-absorbance peak and converting it into μg DNA by means of a calibration curve. Normal (semiconservative) DNA synthesis (cpm/μg DNA) was determined from the gradient profiles as radioactivity incorporated into dense DNA.

A test substance producing a reproducible and significant dose-related increase in radiolabel incorporation is reported as genotoxic. A test substance producing neither a significant, dose-related increase in ^3H incorporation nor a reproducible positive response at any test point is considered non-genotoxic in this test system.

4. Results

4.1 Toxicity

A rough estimate of the cytotoxicity of the test extract for the hepatocyte cultures can be obtained from the degree of inhibition of replicative DNA synthesis (cpm/μg DNA), i. e. from the reduction in the incorporation of radioactivity in semiconservatively replicated (dense) DNA strands. Replicative DNA synthesis was affected at the highest concentration tested (table). As well, test compound showed precipitation at the highest concentration (table).

4.2 Genotoxicity

Testing was done with 6 concentrations of the test compound (0.2-11.7 mM). In both experiments GLYPHOSATE did not induce any increase in DNA repair synthesis (cpm/μg DNA) above control level in the tested concentration range (table). Variations of individual results relative to the controls by $\pm$ 10 % are well within the variability of the control values within individual experiments. In contrast, the positive control chemicals, 2-AAF and DMN, caused a clear enhancement in DNA repair confirming the responsiveness and metabolic activity of the test system (table).

5. Conclusions

The method applied in this study for the determination of DNA repair synthesis in rat hepatocyte primary cultures, the socalled bromodeoxyuridine density-shift method, is based on a technique developed by Pettijohn and Hanawalt (4) for the measurement of the re-synthesis step during excision repair of damaged DNA. Cells are incubated in the presence of the heavy thymidine analog, 5-bromodeoxyuridine (BrdUrd), radioactive deoxycytidine (^{3}H-dCyd), and the test chemical. DNA strands synthesized during the incubation time by normal replicative DNA synthesis get incorporated large amounts of the heavy BrdUrd and, thus, obtain a higher density than unreplicated DNA strands have. If the test chemical causes damage in unreplicated DNA which is subject to excision repair, short stretches of newly synthesized DNA are incorporated containing both BrdUrd and ^{3}H-dCyd. The incorporated BrdUrd is not sufficient, however, to increase significantly the density of the DNA strands. Repaired and replicated DNA strands can subsequently be separated on alkaline cesium salt gradients and quantitatively determined by measuring the incorporated radioactivity.

In the present investigation GLYPHOSATE was tested in rat hepatocytes for its potential to induce genotoxic effects in primary rat hepatocytes. The tested concentration range covered nontoxic and toxic concentrations of the test compound.

In neither of the two experiments performed GLYPHOSATE significantly increased the level of DNA repair above control level. In contrast, the positive control chemicals, dimethylnitrosamine and 2-acetamidofluorene, markedly enhanced DNA repair, demonstrating that both cell viability and drug metabolizing enzyme activities were well preserved in the hepatocyte preparations. Therefore, the present findings clearly indicate that GLYPHOSATE is not genotoxic under conditions described in this test report.

[redacted]

Biological Safety Testing

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6. Table

Table: DNA repair test with primary rat hepatocytes
Test: 931564
Test compound: GLYPHOSATE

	Repair synthesis (cpm/µg DNA)		Replic. synthesis (cpm/µg DNA)	
	Test 1	Test 2	Test 1	Test 2
Negative controls				
WME	105.1	102.8	2322.2	2833.9
	120.3	88.1	2562.2	2313.4
Positive controls				
DMN (10 mM)	973.7	928.5	1794.9	1521.8
2-AAF (0.2 mM)	817.4	728.5	306.2	829.9
Test compound				
0.20 mM	114.8		1998.4	
0.61 mM	109.4		2363.0	
1.14 mM		96.2		2784.2
1.81 mM	115.1		2641.0	
3.41 mM		88.5		2514.7
5.44 mM	102.4		2509.1	
10.23 mM		95.7		2902.6
16.32 mM	109.9		2356.6	
30.69 mM		87.8		3175.0
48.98 mM	98.2		1729.1	
92.08 mM		71.6		2664.6
111.69 mM		66.9		737.2

7. References

1. Rossberger, S. and Andrae, U.: DNA repair synthesis induced by N-hydroxyurea, aceto-hydroxamicacid and N-hydroxyurethane in primary rat hepatocyte cultures: comparative evaluation using the autoradiographic and the bromodeoxyuridine density-shift method. Mutation Res. 145, 201 - 207,1985.

2. Rossberger, S. and Andrae, U.: Background DNA repair synthesis in rat hepatocyte cultures used for genotoxicity testing. Toxic. in Vitro 1, 215 - 223, 1987.

3. Leibhold, L.R. Schwarz: Inhibition of intracellular communication in rat hepatocytes by phenobarbital, 1,1,1-trichloro-2,2-bis(p(chlorophenyl)ethane (DDT) and gamma-hexachlorocyclohexane (lindane): modification by antioxidants ansd inhibitors of cyclo-oxygenase, Carcinogenesis 14 (11), 2377-2382, 1993.

4. Pettijohn, D. E., and Hanawalt, P. C.: Evidence for repair replication of ultraviolet damaged DNA in bacteria. J. Mol. Biol. 9, 395 - 410, 1964.

5. Organisation for economic co-operation and development: Guidelines for testing of chemicals, Genetic toxicology: DNA Damage and repair/unscheduled DNA synthesis in mammalian cells in vitro, Guideline 482 (1986)

6. Grundsätze der Guten Laborpraxis (GLP): Gesetz zum Schutz vor gefährlichen Stoffen (Chemikaliengesetz), BGBl I (1991).

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STATEMENT OF QUALITY ASSURANCE (GLP)

TEST 931564

94-03-31

Test	DNA repair test with primary rat hepatocytes (SOP 9.20, OECD Guideline 482)
Test compound	GLYPHOSATE

This report has been audited by the Quality Assurance and the result has been reported to the study director and the management of the ANAWA München AG on March 31, 1993. Within the course of the regularly conducted self-inspections the Quality Assurance convinced of the compliance with the test instructions and the standard operating procedures during conduction of this study.

[redacted]

Quality Assurance

Bayerisches Staatsministerium für Arbeit, Familie und Sozialordnung

8000 München 40, Winzererstraße 9, Telefon (089) 1261-01

GLP – Bescheinigung

Bescheinigung

Hiermit wird bestätigt, daß die Prüfeinrichtung(en)

Biologisch-chemische Laboratorien

8033 Planegg

in ..... (Ort, Anschrift)

Behringstr. 6

der ANAWA München AG (Firma)

am 23./24.04.1991 (Datum)

von der für die Überwachung zuständigen Behörde über die Einhaltung der Grundsätze der Guten Laborpraxis inspiziert worden ist (sind).

Es wird hiermit bestätigt, daß folgende Prüfungen in dieser Prüfeinrichtung nach den Grundsätzen der Guten Laborpraxis durchgeführt werden.

Certificate

It is hereby certified that the test facility(ies)

..... (location, address)

in .....

of ..... (company name)

on ..... (date)

was (were) inspected by the competent authority regarding compliance with the Principles of Good Laboratory Practice.

It is hereby certified that studies in this test facility are conducted in compliance with the Principles of Good Laboratory Practice.

Die Prüfungen von Stoffen und Zubereitungen betreffen:

- Pharmakologische Analytik:
  Prüfung von Wirkstoffgehalt, -stabilität und -rückständen in Wirkstoffpräparaten biologischer Proben
- Toxikologische Prüfungen an Zellkulturen:
  Zytotoxizitäts-, Mutagenitätstests
- Wirksamkeitsprüfungen an Zellkulturen:
  Bestimmung der biologischen Aktivität
- Biologische Sicherheitsprüfungen:
  Stabilitätstest, Keimzahlbestimmung, Pyrogentest

München, den 20.03.1992

[redacted]

Dipl.-Chem.
Ministerialrat

L.S.

Bundesgesundheitsamt

- GLP-Bundesstelle -

GUTE LABORPRAXIS
GOOD LABORATORY PRACTICE

GLP-Bescheinigung / Statement of Compliance

(gemäß / according to § 19b Abs.2 Nr.3 Chemikaliengesetz)

Eine GLP-Inspektion wurde durchgeführt in / A GLP inspection was carried out at

Prüfeinrichtung / Test facility

RALLIS INDIA LIMITED
Agrochemical Research Station
Bangalore 560 058
India

Prüfkategorien / Area of Expertise

Prüfungen auf toxikologische Eigenschaften an Ratte, Maus, Kaninchen und Vogel
Toxicity studies with rat, mouse, rabbit and bird

Datum der Inspektion / Date of Inspection

30.03.-02.04.1992

Auf der Grundlage des Inspektionsberichtes und der Besprechung über zu erfolgende Maßnahmen wird hiermit bestätigt, daß in dieser Prüfeinrichtung die obengenannten Prüfungen zum Zeitpunkt der Inspektion unter Einhaltung der GLP-Grundsätze durchgeführt wurden.

Based on the inspection report and the discussion of follow up activities it can be confirmed, that at time of inspection the test facility were conducting the aforementioned studies in compliance with the Principles of Good Laboratory Practice.

27. October 1992

In [redacted]

BUNDESGESUNDHEITSAMT

Leiter GLP-Bundesstelle
GLP Federal Office