Mutation Research 721 (2011) 89–94

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Phenylurea herbicides induce cytogenetic effects in Chinese hamster cell lines

Concetta Federico[a], Salvatore Motta[a], Cristina Palmieri[a], Matteo Pappalardo[b], Vito Librando[b,c], Salvatore Saccone[a,c,*]

[a] Dipartimento di Biologia Animale "M. La Greca", University of Catania, via Androne 81, I-95124 Catania, Italy
[b] Dipartimento di Scienze Chimiche, University of Catania, viale A. Doria 6, Catania, Italy
[c] Centro Universitario di Analisi, Monitoraggio e Metodologie di Minimizzazione del Rischio Ambientale c/o Dipartimento di Scienze Chimiche, University of Catania, viale Andrea Doria, 6, 95125 Catania, Italy

ARTICLE INFO

Article history:
Received 6 August 2010
Received in revised form
23 November 2010
Accepted 27 December 2010
Available online 14 January 2011

Keywords:
In vitro genotoxic tests
Chromosomal aberrations
Sister chromatid exchange
Mammalian cells
Herbicides

ABSTRACT

The intensive use of herbicides over the last few decades has caused a general increase of environmental pollution. It is thus very important to evaluate the possible genotoxic properties of these chemical compounds as well as identifying their mode of action. Phenylurea herbicides are selective agents widely used for the control of infestant plants. Of these herbicides, which are widely used in agriculture, we analysed four of the less intensively studied molecules. More precisely, we investigated the genotoxic effects of fenuron, chlorotoluron, diuron, and difenoxuron by analyses of chromosomal aberrations (CAs) and sister chromatid exchange (SCE) in exposed mammalian cells. We used the Chinese hamster ovary (CHO) and epithelial liver (CHEL) cell lines, endowed with the absence or the presence, respectively, of an enzymatic system to activate pro-mutagenic compounds. Our results show that all herbicides tested induce, at high concentrations, an increasing number of CAs in non-metabolising CHO cells. Instead, in the exposed CHEL cell line, the four herbicides induced CAs also at the lowest dose-level. In the CHEL cells, a statistically significant increase of SCE was also observed. The phenylurea herbicides showed direct genotoxic activity, but the cytogenetic effects were greatly enhanced after metabolic conversion. These data, together with other information on phenylurea herbicides, are of great interest from the environmental point of view, and for human health. In fact, intensive use of herbicides contaminates soil, surface water, groundwater and agricultural products, and thus should be taken in particular consideration not only for those initiatives to specifically protect exposed workers, but also to safeguard the health of consumers of agricultural products.

1. Introduction

The pesticides that are currently used in agriculture include a number of pollutants that have been grouped in different classes, considering their chemical features, mode of action, and the possibility of being absorbed [1]. A specific class of pesticides is that of the herbicides or weed-killers, which could be selective or not selective, depending on their toxic effect only on some particular species or on all plant species [1,2]. Some herbicides are able to cross the phospolipid membrane of leaves, while others, when released into the soil, are resistant to degradative mechanisms through chemicophysical processes such as pH, ion strength, and then penetrate into plants by their roots. Most of the more recent herbicides are sprinkled on the infested plants during early development, and then interfere with their growth with no damage to the farm crops [3,4]. In fact, fully non-selective weed-killers, such as paraquat, ammonium glufosinate, and glifosate can only be used before seedling germination. To improve weed-killers and to promote a wider use of those with a non-selective mode of action, new plant varieties that are resistant to non-selective herbicides have been recently produced [5,6].

In vitro mutagenic short-term assays are widely used to identify putative mutagenic activity of a variety of chemical compounds. Pesticides are largely used in agriculture, and a number of these have mutagenic activity [7–9]. Thus, genotoxic assays of specific pesticides are very important especially to protect the health of people working in these areas, who are exposed via inhalation, skin contact, or ingestion to high amounts of these compounds.

To evaluate the mutagenic properties of specific chemicals, a number of different *in vitro* tests have been developed, some of which involve the use of mammalian cells grown in the presence of an external metabolising system, normally a sub-cellular S9-fraction from rodent liver able to activate chemical carcinogens [10]. However, many efforts have been made to establish cultures of metabolically active target cells for the detection of

* Corresponding author at: Dept. Biologia Animale "M. La Greca", Via Androne, 81, I-95124 Catania, Italy. Tel.: +39 095 7306036; fax: +39 095 327990.
E-mail address: saccosal@unict.it (S. Saccone).

1383-5718/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2010.12.013

Table 1
Chromosome aberrations in a Chinese hamster ovary (CHO) cell line.

	Dose-level μg/ml	CA/cell		Ab. cells %	Mitotic index (%)	Statistical significance
		Chromatid type	Chromosome type			
Fenuron	1.10	0.050	0.005	5.0	7.4	NS
	11.00	0.050	0.010	5.0	7.4	NS
	110.00	0.065	0.010	7.5	7.0	$P<0.05$
	220.00	0.095	0.010	9.0	6.3	$P<0.01$
Chlorotoluron	0.48	0.035	0.015	5.0	6.2	NS
	4.80	0.040	0.010	5.0	7.4	NS
	48.00	0.080	0.000	8.0	6.6	$P<0.05$
	96.00	0.075	0.015	8.5	5.6	$P<0.05$
Diuron	0.45	0.060	0.005	6.5	9.1	NS
	4.50	0.080	0.010	8.5	6.7	$P<0.05$
	45.00	0.105	0.020	10.5	7.0	$P<0.05$
	90.00	0.170	0.020	16.5	6.1	$P<0.0001$
Difenoxuron	0.95	0.040	0.015	5.5	7.1	NS
	9.50	0.055	0.010	6.5	6.9	NS
	95.00	0.050	0.025	7.0	7.3	NS
	190.00	0.075	0.010	8.0	6.0	$P<0.05$
Negative control	Untreated cells	0.021	0.010	3.1	8.2	–
Negative control	Acetone	0.040	0.013	4.9	7.8	NS
Positive control	MM-C	1.079	0.093	69.1	3.4	$P<0.0001$

CA/cell: chromosome aberrations per cell; Ab. cells %: percentage of cells with aberrations; NS: statistically not significant; MM-C: mitomycin-C. Negative and positive control data are averages from all the performed tests. Statistical significances were evaluated with respect to the negative controls performed in each test.

genotoxic events [11–14]. A near-diploid Chinese hamster epithelial liver (CHEL) cell line [15,16], which retains a considerable level of metabolising enzyme activity [17], has been used in short-term tests for mutagenic and cytogenetic effects, in which the same cell acts both as a metabolic source and as a target for genotoxic damage [18,19].

Here we show the results of the evaluation of genotoxic effects on four phenylurea herbicides by analyses of chromosomal aberrations (CAs), and sister chromatid exchange (SCE) on exposed mammalian Chinese hamster ovary (CHO), and epithelial liver (CHEL) cell lines, endowed with the absence or the presence, respectively, of an enzymatic system able to activate a number of pro-mutagenic compounds. Phenylurea herbicides, belonging to the C2 group of the Herbicide Resistance Action Committee (HRAC) classification, with a mode of action that determines inhibition of photosynthesis at the photosystem II complex [3,4,20] were tested. More precisely, we analysed the following phenylurea herbicides (Fig. 1): (i) *fenuron*, commercially known as Dybar, a non-selective herbicide recommended for the control of woody plants, and against the germination of infestant grasses. It is also used when planting leeks, onions, fruit trees, and in garden centres; (ii) *chlorotoluron*, commercially known as Dicuran, used to protect graminae, especially wheat and barley; (iii) *diuron*, which shows a wide spectrum of action and is generally used as a weed-killer in tree cultures, asparagus, flowers, paddy fields, and to protect industrial areas from infestant plants. Diuron is also commercially known as Karmex; (iv) *difenoxuron*, commercially known as Lironion, prevalently used for onions. In the soil, physical, chemical, and microbial agents easily degrade difenoxuron.

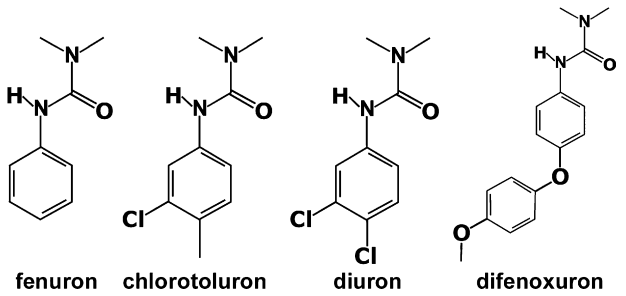

Fig. 1. Chemical structures of the phenylurea herbicides used in the present work.

2. Materials and methods

2.1. Chemicals

Fenuron: 1,1-dimethyl-3phenylurea (CAS no. 101-42-8), *chlorotoluron*: 3-(3-chloro-*p*-tolyl)-1,1-dimethylurea (CAS no. 15545-48-9), *diuron*: 3-(3,4-dichlorophenyl)-1,1-dimethylurea (CAS no. 330-54-1), *difenoxuron*: 3-[4-(4-methoxyphenoxy)phenyl]-1,1-dimethylurea (CAS no. 14214-32-5), were obtained from Riedel-de Haen (Hannover, Germany)

2.2. Cell cultures and chromosome preparations

Cultures of CHO and CHEL cell lines were prepared from frozen stocks for experimental use. They were routinely cultured in William's medium supplemented with 10% foetal calf serum (FCS), 50 UI/ml penicillin, and 50 UI/ml streptomycin (all cell-culture media and reagents were from Gibco-Invitrogen, USA). Cells were grown at 37 °C in a 5% CO_2 atmosphere.

Metaphase chromosomes were prepared following standard cytogenetic procedures. Briefly, colcemid was added for the last 3 h at a concentration of 0.05 μg/ml, and then cells were detached by trypsinization. The cell suspension was centrifuged and re-suspended in hypotonic solution (1% sodium citrate, 20 min). After hypotonic treatment cells were fixed in freshly prepared methanol–acetic acid (3:1). Chromosome spreads were prepared on air-dried slides, ready to use for the subsequent staining procedures to detect SCE or CAs (see below).

2.3. Herbicide preparation for cytogenetic tests

Fenuron, chlorotoluron, diuron, and difenoxuron were dissolved in acetone (Mallinckrodt Baker, Deventer, Holland) at the highest possible concentration on the basis of the solubility of each herbicide [2,21]. A stock solution of each herbicide was prepared at the concentration of 110 mg/ml, 48 mg/ml, 45 mg/ml, and 95 mg/ml, respectively. From the above stock solution, four dose-levels of each herbicide were used. Ten μl of each dose-level were added to 5 ml cell culture, to obtain the final concentrations indicated in Tables 1 and 2, and an amount of solvent not exceeding 0.2%.

Negative and positive controls were also included in the experiment. The negative controls consisted of both untreated and solvent-treated cultures. The positive controls were mitomycin-C (0.10 μg/ml), and 7,12-dimethylbenz(*a*)anthracene (4 μg/ml) for the CHO and CHEL cell lines, respectively [22]. As it has been shown that factors such as pH and osmotic pressure could cause genetic damage [23] they were determined following treatments. The reaction mixtures had no effects on either of these factors, which remained within physiological limits.

Table 2
Chromosome aberrations in a Chinese hamster epithelial liver (CHEL) cell line.

	Dose-level μg/ml	CA/cell		Ab. cells %	Mitotic index (%)	Statistical significance
		Chromatid type	Chromosome type			
Fenuron	1.10	0.180	0.045	18.5	7.3	$P < 0.0001$
	11.00	0.385	0.040	34.5	7.5	$P < 0.0001$
	110.00	0.425	0.070	39.0	7.1	$P < 0.0001$
	220.00	0.525	0.080	45.5	6.2	$P < 0.0001$
Chlorotoluron	0.48	0.075	0.010	07.5	6.1	$P < 0.01$
	4.80	0.100	0.030	11.5	7.3	$P < 0.0001$
	48.00	0.235	0.020	19.5	6.7	$P < 0.0001$
	96.00	0.270	0.045	23.5	5.9	$P < 0.0001$
Diuron	0.45	0.185	0.040	21.5	9.2	$P < 0.05$
	4.50	0.215	0.020	18.0	6.8	$P < 0.01$
	45.00	0.330	0.050	30.0	7.3	$P < 0.0001$
	90.00	0.405	0.040	33.0	6.1	$P < 0.0001$
Difenoxuron	0.95	0.145	0.030	17.5	7.2	$P < 0.0001$
	9.50	0.230	0.040	23.0	7.0	$P < 0.0001$
	95.00	0.320	0.035	31.0	7.3	$P < 0.0001$
	190.00	0.455	0.045	40.0	6.0	$P < 0.0001$
Negative control	Untreated cells	0.028	0.011	3.5	8.2	–
Negative control	Acetone	0.061	0.013	7.0	7.7	NS
Positive control	DMBA	1.051	0.119	51.1	3.4	$P < 0.0001$

CA/cell: chromosome aberrations per cell; Ab. cells %: percentage of cells with aberrations; NS: statistically not significant; DMBA: 7,12-dimethylbenz(*a*)anthracene. Negative and positive control data are averages from all the performed tests. Statistical significances were evaluated with respect to the negative controls performed in each test.

2.4. Assay procedures

For the chromosomal aberration assay (CAs), approximately 24 h before each experiment, 25-cm^2 flasks were seeded with 300,000 cells to ensure that cells were in the exponential growth phase at the time of treatment with the herbicides. Two cultures for each dose-level were established, and treatments were conducted for 20 h. Colcemid was added during the last 3 h, and after harvesting and slide preparations (see above), the chromosomes were stained in 4% Giemsa.

For the SCE assay, the flasks with cell cultures in the exponential growth phase were prepared as above and herbicides, dissolved in acetone, were added at the appropriate dose-levels. Afterwards, bromodeoxyuridine (BrdUrd) was added to each flask at a final concentration of 10 μg/ml, and incubated under safety light until harvesting 26 h later. Chromosome staining for visualization of SCE was performed by use of the FPG method, as previously described [24].

2.5. Evaluation of results

Slides were randomly assigned code numbers by a person not subsequently involved in slide evaluation. Cytogenetic analysis of slides was undertaken blind with coded slides. Evaluation of mitotic index (MI) was based on the number of metaphases observed per 1000 cells and was expressed as a percentage.

At least 200 and 50 metaphases were scored per test point in the CA and SCE tests, respectively. CAs were classified as chromatid-type gaps, chromatid-type breaks, chromatid-type exchanges, chromosome-type gaps, chromosome-type breaks, chromosome-type exchanges and iso-locus events, which include isochromatid and iso-locus breaks when these cannot be distinguished, as described previously [25].

To determine statistical significance, Fisher's exact test was used. For the SCE assays we evaluated the increase in the mean value of SCE in at least 50 randomly selected metaphases. The statistical significance for the SCE assays was determined by use of the two-tailed *T*-test.

3. Results

The four phenylurea herbicides fenuron, chlorotoluron, diuron, and difenoxuron (Fig. 1) were studied to evaluate their mutagenic properties by chromosomal aberration (CA), and sister chromatid exchange (SCE) tests. The CA results obtained in CHO and CHEL cell lines exposed to the above herbicides are presented in Tables 1 and 2, respectively, in which the frequency of chromatid and chromosome aberrations, and the percentage of cells bearing aberrations (% aberrant cells) – excluding gaps – are reported for each dose level. A summarizing scheme of the CA data for both cell lines for each herbicide tested is shown in Fig. 2. SCE assays were performed on CHEL cells exposed to the four phenylurea herbicides, and the average number of SCE (with the relative confidence interval) for each dose level, are presented in Fig. 3.

Treatment with herbicides caused only a very slight toxicity to the cells. At the highest doses, the mitotic indices (MIs) were never reduced by more than 65% of the control values. As outlined in the most representative guidelines for testing chemicals and their capability to induce chromosomal damage, the highest dose-level used for the scoring of aberrations should be a concentration that causes moderate toxicity (ideally the reduction in MI should be approximately 50% of the control value). Treatments reducing the MI below 20% should not be considered. On the basis of the MIs from the present results, all the treatment dose-levels were used for scoring both CAs and SCE.

3.1. Chromosome-aberration test

A general increase in the percentage of aberration-bearing cells compared with the relevant solvent-control values was observed in treated cultures. Statistical significance was achieved for the CHEL cell lines for every herbicide treatment at all dose-levels tested. The effect exceeded the mean historical control value for aberrant cells – excluding gaps – recorded in our laboratory for this cell line. Moreover, a marked increase in the frequency of aberrant cells was seen in the cultures with the positive control substance, indicating the correct functioning of the assay system. In the case of CA tests in the non-metabolising CHO cell line, the results clearly indicate that phenylurea herbicides induce aberrations, generally at higher doses.

In detail, for each herbicide, we obtained the following results:

- *Fenuron*: in the CHO cell line, at the lower dose levels no statistically significant increase of CAs was observed. Instead, the 110- and 220-μg/ml doses caused a significant increase in CAs. In the CHEL cell line, a very high, statistically significant increase of CAs was observed also at the lower dose level, corresponding to 1.1 μg/ml of this herbicide.
- *Chlorotoluron*: in the CHO cell line, also with this herbicide at the lower doses, no statistically relevant increase of CAs was observed compared with the controls. However, at the higher dose levels a significant increase of CAs was seen. In this case, the higher doses

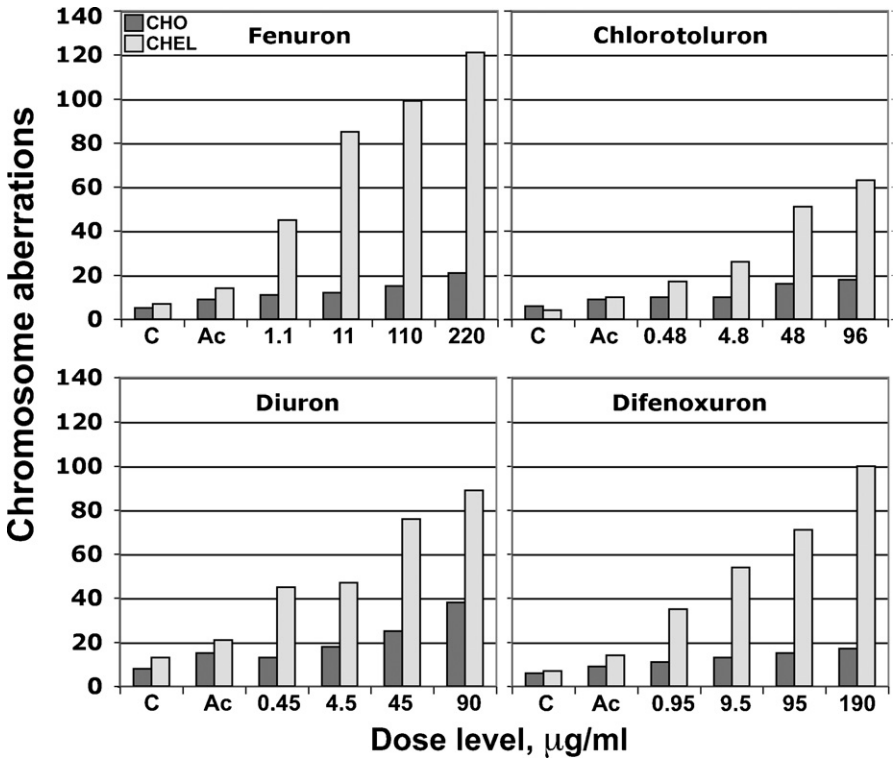

Fig. 2. Chromosome aberrations (CAs) induced by the indicated phenylurea herbicides in exposed CHO and CHEL cells. C: untreated controls, Ac: controls with acetone. Dose levels are in μg/ml. CAs were scored in 200 cells.

are lower than those of fenuron, *viz.* 48 and 96 μg/ml. In the CHEL cell line, *chlorotoluron* induced a very high, statistically significant increase of CAs also at the lower dose, corresponding, in this case, to 0.48 μg/ml of herbicide.

- *Diuron*: only the lowest dose level (0.45 μg/ml) did not induce CAs in CHO cells, a statistically significant increase of CAs was observed at the 4.5-μg/ml dose. The CA test with the CHEL cell line exposed to diuron showed an increased number of aberrations for all doses tested and a clear dose-effect correlation.
- *Difenoxuron*: in the CHO cell line, this herbicide showed a statistically significant increase of CAs only at the highest dose (190 μg/ml), and no statistically significant change at the other three dose levels. Instead, in the CHEL cell line the induction of CAs was statistically significant at each dose.

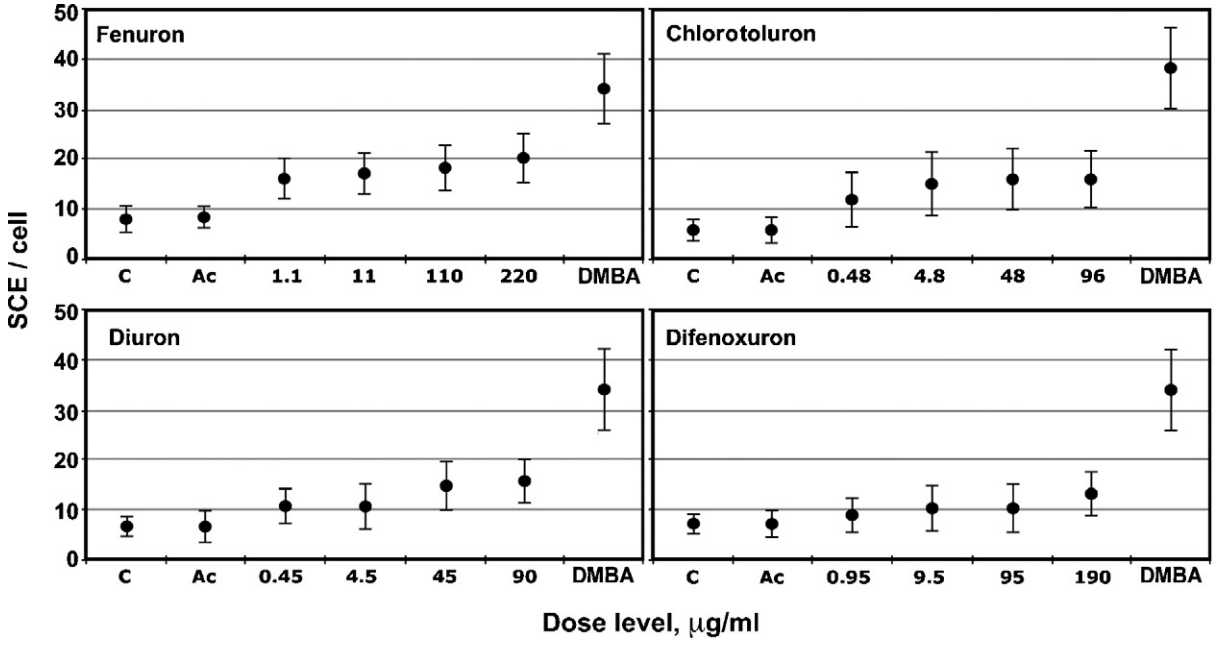

Fig. 3. SCE induced by the indicated phenylurea herbicides on exposed CHEL cells. C: untreated controls, Ac: controls with acetone. Dose levels are in μg/ml.

3.2. Sister chromatid exchange test

SCE were evaluated in the CHEL cell line, and, in general, a statistically significant increase was observed compared with the controls, for every dose level and for all herbicides tested. Fenuron showed the highest level of induction also at the lower dose (1.1 μg/ml), with a very high, statistically significant number of SCE compared with the controls. Difenoxuron showed a good dose-effect correlation, with the lowest and the highest number of SCE observed with the lowest (0.95 μg/ml) and the highest (190 μg/ml) dose, respectively (Fig. 3).

4. Discussion

Intensive use of herbicides determines a general increase of environmental pollution, especially in the rural areas where these compounds are widely employed. Here, we studied the genotoxic properties of the four phenylurea herbicides fenuron, chlorotoluron, diuron, and difenoxuron. There are very few studies, and not all of them concordant, on the genotoxic features of the phenylurea herbicides tested here, and, generally, these data were limited to diuron. This latter herbicide was described as 'suspect genotoxic', with the Mutatox test with the dark variant (M-169) of *Photobacterium phosphoreum*, directly and after S9 activation [26], but not mutagenic in *Salmonella typhimurium* with or without activation [27]. More recently, compared with controls a significantly higher level of aneuploidy was observed in Pacific oysters *Crassostrea gigas* exposed to diuron [28].

In the present work, we studied the mutagenic properties of the above-mentioned phenylurea herbicides by measuring their cytogenetic effects on exposed CHO and CHEL cell lines. Comparison of data obtained with non-metabolising CHO and metabolically competent CHEL cells provides information on the mutagenic or pro-mutagenic properties of the test chemicals. Our investigation with the phenylurea herbicides was performed by analysis of two cytogenetic biomarkers, chromosomal aberrations (CAs) and sister chromatid exchange (SCE), as these tests are widely used to identify the genotoxic properties of a variety of chemical compounds [18,29–32] not only with Chinese hamster cells, but also by use of a number of other mammalian cell types, including human lymphocytes [7,33,34].

The present results show that each of the test herbicides is characterized by a direct genotoxic activity, as is demonstrated by the increasing number of CAs in non-metabolising CHO cells, which was seen at the highest dose levels. More precisely, compared with controls a statistically significant increase of CAs was observed starting from the dose of 4.50 μg/ml, 48 μg/ml, 110 μg/ml, and 190 μg/ml for diuron, chlorotoluron, fenuron, and difenoxuron, respectively. These data indicate that these four phenylurea herbicides are endowed with direct mutagenic properties, with some differences in the amount of herbicide required to display this property. In fact, diuron was able to induce CAs in CHO cells already at a low concentration (4.50 μg/ml) in contrast to the other compounds, which only induced CAs at higher doses, with difenoxuron showing a statistically significant induction ($P < 0.05$) of CAs only at the highest dose (190 μg/ml), i.e. about 40 times higher than that of diuron.

Data obtained with the metabolising CHEL cell line show a high level of CAs and SCE at all dose levels tested, also including herbicide amounts below 1 μg/ml. In this case, fenuron was the herbicide with the strongest cytogenetic effect, showing a very high, statistically significant level ($P < 0.001$) of CAs and SCE at every dose, starting from 1.1 μg/ml. This indicates the presence of additional derived compounds with genotoxic properties. Thus, the stronger cytogenetic effects induced at every dose level after metabolic activation of the four herbicides could be explained by the sum of (i) the intrinsic direct mutagenicity and (ii) the mutagenic properties of intermediate metabolites produced in the CHEL cells. This is in agreement with a variety of intermediate compounds identified for some of the herbicides tested, such as 2,4-dichloroaniline (DCA), which in CHEL cells could exert its putative genotoxic effect due to its formation directly inside the cell, despite its being described as being only weakly genotoxic in the Ames test [35] and rapidly photo-degraded in water after exposure to solar light [2].

Thus, the four phenylurea herbicides fenuron, chlorotoluron, diuron, and difenoxuron could be considered as direct mutagens, even if they generally exert – with the exception of diuron – their mutational property at high concentrations. Taking into consideration the data obtained with CHEL cells, where statistically significant increases of cytogenetic effects were observed at every dose level, including the lowest, these herbicides could be indicated as pro-mutagens, i.e. they are metabolised in CHEL cells into intermediate compounds that enhance the number of chromosomal alterations observed as CAs and SCE. Finally, we can assert the usefulness of the test system employed, consisting of the use of two different cell lines with different ways of metabolising xenobiotics. In this study, this test system for genotoxicity allowed us to identify the cytogenetic effects of a group of herbicides belonging to the same chemical family.

Our data, together with other information on phenylurea herbicides, could be useful not only from the environmental point of view, namely in controlling and helping to plan specific measures aimed at reducing the environmental hazards from exposure to these compounds, but also for human health. In fact, the intensive use of herbicides contaminates soil, surface water, groundwater and agricultural products, and particular care should be taken to specifically protect exposed agricultural workers and consumers of herbicide-treated products. Further studies should be focused on the interaction of such pesticides with small molecules present in the environment, or on the effect of chemical–physical parameters with respect to their degradation and in their alteration of biological properties such as genotoxicity.

Conflict of interest statement

The authors declare no conflict of interests.

Acknowledgement

The work was supported by PRA from the University of Catania to S.S. and V.L.

References

[1] J.R. Reigart, J.R. Roberts, Recognition and Management of Pesticide Poisonings, fifth ed., 1999, Available at: http://www.epa.gov/oppfead1/safety/healthcare/handbook/handbook.htm.
[2] C.R. Worthing, R.J. Hance (Eds.), The Pesticide Manual: A World Compendium, 10th ed., British Crop Protection Council, Surrey, United Kingdom, 1995.
[3] S.O. Duke, Overview of herbicide mechanisms of action, Environ. Health Perspect. 87 (1990) 263–271.
[4] J.E. Casida, Pest toxicology: the primary mechanisms of pesticide action, Chem. Res. Toxicol. 22 (2009) 609–619.
[5] S.B. Powles, Evolved glyphosate-resistant weeds around the world: lessons to be learnt, Pest. Manag. Sci. 64 (2008) 360–365.
[6] T.A. Gaines, W. Zhang, D. Wang, B. Bukun, S.T. Chisholm, D.L. Shaner, S.J. Nissen, W.L. Patzoldt, P.J. Tranel, A.S. Culpepper, T.L. Grey, T.M. Webster, W.K. Vencill, R.D. Sammons, J. Jiang, C. Preston, J.E. Leach, P. Westra, Gene amplification confers glyphosate resistance in Amaranthus palmeri, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 1029–1034.
[7] C. Bolognesi, Genotoxicity of pesticides: a review of human biomonitoring studies, Mutat. Res. 543 (2003) 251–272.
[8] S. Bull, K. Fletcher, A.R. Boobis, J.M. Battershill, Evidence for genotoxicity of pesticides in pesticide applicators: a review, Mutagenesis 21 (2006) 93–103.
[9] M.C. Alavanja, M.H. Ward, P. Reynolds, Carcinogenicity of agricultural pesticides in adults and children, J. Agromedicine 12 (2007) 39–56.

[10] B.N. Ames, J. McCann, E. Yamasaki, Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test, Mutat. Res. 31 (1975) 347–364.
[11] B.J. Dean, G. Hodson-Walker, An *in vitro* chromosome assay using cultured rat-liver cells, Mutat. Res. 64 (1979) 329–337.
[12] P.T. Iype, S. Turner, M.S. Siddiqi, Markers for transformation in rat liver epithelial cells in culture, Ann. N.Y. Acad. Sci. 349 (1980) 312–322.
[13] B.L. Allen-Hoffmann, J.G. Rheinwald, Polycyclic aromatic hydrocarbon mutagenesis of human epidermal keratinocytes in culture, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 7802–7806.
[14] N. Danford, P. Wilcox, J.M. Parry, The clastogenic activity of dinitropyrenes in a rat-liver epithelial cell line, Mutat. Res. 105 (1982) 349–355.
[15] G. Turchi, M.A. Carluccio, F. Oesch, I. Gemperlein, H.R. Glatt, Characterisation of an epithelial nearly diploid liver cell strain from Chinese hamster able to activate promutagens, Mutagenesis 2 (1987) 127–135.
[16] G. Turchi, A. Nardone, F. Palitti, Application of an epithelial cell line metabolically competent for mutation studies of promutagens, Mutat. Res. 271 (1992) 79–88.
[17] J.W. Grisham, R.K. Charlton, D.G. Kaufman, *In vitro* assay of cytotoxicity with cultured liver: accomplishments and possibilities, Environ. Health Perspect. 25 (1978) 161–171.
[18] S. Motta, C. Federico, S. Saccone, V. Librando, P. Mosesso, Cytogenetic evaluation of extractable agents from airborne particulate matter generated in the city of Catania (Italy), Mutat. Res. 561 (2004) 545–552.
[19] R. De Salvia, R. Meschini, M. Fiore, S. Polani, F. Palitti, M.A. Carluccio, G. Turchi, Induction of sister chromatid exchanges by procarcinogens in metabolically competent Chinese hamster epithelial liver cells, Mutat. Res. 207 (1988) 69–75.
[20] J. Liu, Phenylurea herbicides, in: R.I. Krieger (Ed.), Handbook of Pesticide Toxicology: Amount of a Urea Herbicide has been Ingested Within 1 hour, there Principles, 2nd ed., Academic Press, San Diego, CA, 2001, pp. 1521–1527.
[21] D. Hartley, H. Kidd (Eds.), The Agrochemicals Handbook, 2nd ed., The Royal Society of Chemistry, Lechworth, Herts, England, 1987, p. A206.
[22] E. Madle, G. Tiedemann, S. Madle, A. Ott, G. Kaufmann, Comparison of S9 mix and hepatocytes as external metabolizing systems in mammalian cell cultures: cytogenetic effects of 7,12-dimethylbenzanthracene and aflatoxin B1, Environ. Mutagen. 8 (1986) 423–437.
[23] A.H. Seeberg, P. Mosesso, R. Forster, High dose-level effects in mutagenicity assays utilizing mammalian cells in culture, Mutagenesis 3 (1988) 213–218.
[24] P. Perry, J. Wolff, New Giemsa method for the differential staining of sister chromatid, Nature 251 (1974) 156–158.
[25] J.R.K. Savage, Classification and relationships of induced chromosomal structural changes, J. Med. Genet. 12 (1975) 103–122.
[26] S. Canna-Michaelidou, A.S. Nicolaou, Evaluation of the genotoxicity potential (by Mutatox test) of ten pesticides found as water pollutants in Cyprus, Sci. Total. Environ. 193 (1996) 27–35.
[27] J.P. Seiler, Herbicidal phenylalkylureas as possible mutagens I. Mutagenicity tests with some urea herbicides, Mut. Res. Genet. Toxicol. 58 (1978) 353–359.
[28] K. Bouilly, M. Bonnard, B. Gagnaire, T. Renault, S. Lapègue, Impact of diuron on aneuploidy and hemocyte parameters in Pacific oyster, *Crassostrea gigas*, Arch. Environ. Contam. Toxicol. 52 (2007) 58–63.
[29] S. Soloneski, M. González, E. Piaggio, M.A. Reigosa, M.L. Larramendy, Effect of dithiocarbamate pesticide zineb and its commercial formulation, azzurro III. Genotoxic evaluation on Chinese hamster ovary (CHO) cells, Mutat. Res. 514 (2002) 201–212.
[30] J. Juchimiuk, J. Maluszynska, Transformed roots of *Crepis capillaris* – a sensitive system for the evaluation of the clastogenicity of abiotic agents, Mutat. Res. 565 (2005) 129–138.
[31] M. Gonzalez, S. Soloneski, M.A. Reigosa, M.L. Larramendy, Genotoxicity of the herbicide 24-dichlorophenoxyacetic and a commercial formulation, 2,4-dichlorophenoxyacetic acid dimethylamine salt. I. Evaluation of DNA damage and cytogenetic endpoints in Chinese Hamster ovary (CHO) cells, Toxicol. In Vitro 19 (2005) 289–297.
[32] N.V. González, S. Soloneski, M.L. Larramendy, Genotoxicity analysis of the phenoxy herbicide dicamba in mammalian cells in vitro, Toxicol. In Vitro 20 (2006) 1481–1487.
[33] F. Mañas, L. Peralta, J. Raviolo, O.H. García, A. Weyers, L. Ugnia, C.M. Gonzalez, I. Larripa, N. Gorla, Genotoxicity of AMPA, the environmental metabolite of glyphosate, assessed by the Comet assay and cytogenetic tests, Ecotoxicol. Environ. Saf. 72 (2009) 834–837.
[34] G. Jovtchev, S. Gateva, M. Stergios, S. Kulekova, Cytotoxic and genotoxic effects of paraquat in Hordeum vulgare and human lymphocytes in vitro, Environ. Toxicol. 25 (2010) 294–303.
[35] C.D. Lyons, S.E. Katz, R. Bartha, Persistence and mutagenic potential of herbicide derived aniline residues in pond water, Bull. Environ. Contam. Toxicol. 35 (1985) 696–703.