



## Tumor promoting and co-carcinogenic effects in medium-term rat hepatocarcinogenesis are not modified by co-administration of 12 pesticides in mixture at acceptable daily intake

Julio Isael Perez-Carreón<sup>a,c</sup>, Celine Dargent<sup>a</sup>, Maysaloun Merhi<sup>a</sup>, Samia Fattel-Fazenda<sup>b</sup>, Evelia Arce-Popoca<sup>b</sup>, Saul Villa-Treviño<sup>b</sup>, Patrick Rouimi<sup>a,\*</sup>

<sup>a</sup>UMR 1089-Xénobiotiques, Institut INRA-ENVT, 180 chemin de Tournefeuille, BP 3, F-31 300 Toulouse, France

<sup>b</sup>Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados (CINVESTAV), Av. IPN 2508 Col. San Pedro Zacatenco 07360 Mexico, DF, Mexico

<sup>c</sup>Instituto Nacional de Medicina Genómica (INMEGEN), Periférico Sur No. 4124, Torre Zafiro II, Piso 6 Col. Ex Rancho de Anzaldo 01900, México, DF, Mexico

### ARTICLE INFO

#### Article history:

Received 3 September 2008

Accepted 8 December 2008

#### Keywords:

Carcinogenicity test  
Pesticide mixture  
Preneoplastic lesion  
Cancer risk  
GSTP tumor marker  
Acceptable daily intake

### ABSTRACT

The purpose of this investigation was to evaluate the possible influence of a mixture of pesticides on medium-term carcinogenesis using improved hepatocarcinogenesis protocols. We performed a 12 commercially available pesticides combination with alachlor, atrazine, carbofuran, chlorpyrifos, diazinon, dicofol, endosulfan, iprodione, mancozeb, maneb, procymidone and rotenone. The mixture was given at 1-fold and 10-fold the acceptable daily intake (ADI) level in a set of Solt–Farber-derived protocols involving diethylnitrosamine, 2-acetylaminofluorene treatments and a partial hepatectomy. Co-carcinogenic effect and promoting activity were evaluated using  $\gamma$ -glutamyl transpeptidase (GGT) positive altered hepatocyte foci, as well, protein and mRNA levels of glutathione S-transferase P (GSTP) in liver extracts as molecular biomarkers of carcinogenic effects. The pesticide treatments when compared to vehicle treatments always produced the same number of hepatocyte lesions and an equal GSTP expression on liver extracts independently of carcinogenic-protocol utilized. On this base, we concluded that the pesticide mixture evaluated in this report does not have tumor promoting activity or co-carcinogenic effect in the rat medium-term liver carcinogenesis. Altogether these data contribute to the confidence that the ADI represents a safe intake level to mixture of pesticides at dietary exposure.

© 2008 Elsevier Ltd. All rights reserved.

### 1. Introduction

One important source of non-occupational human exposure to pesticides is through diet. Although not supported by a body of outcomes from epidemiological and experimental studies, there has been an increase in public concerns that exposure to pesticide residues in food and water might pose a cancer risk to the general population (Ames and Gold, 1997; Gold et al., 2001; Hodgson and Levi, 1996). The joint FAO/WHO meeting on pesticide residues (JMPR) reviews toxicological data of pesticides and estimates toxicological risk for humans. It establishes values such as the acceptable daily intake (ADI) of pesticides and the acute reference doses (ARfD) (Herrman and Younes, 1999). The ADI value is obtained from the no-observable adverse effect level (NOAEL) of single mol-

*Abbreviations:* ADI, acceptable daily intake; GGT,  $\gamma$ -glutamyl transpeptidase; GSTP, glutathione S-transferase P; NOAEL, non-observed adverse effects level; HCC, hepatocellular carcinoma; DEN, diethylnitrosamine; 2AAF, 2-acetylaminofluorene; PH 70%, partial hepatectomy; AHF, altered hepatocyte foci.

\* Corresponding author. Tel.: +33 0 561285393; fax: +33 0 561285244.

E-mail address: [prouimi@toulouse.inra.fr](mailto:prouimi@toulouse.inra.fr) (P. Rouimi).

ecule testing in animal experiments, generally divided by a safety factor of at least 100 taking into account intra- and inter-species variability. ADI is interpreted as a safe intake level in individual long-term exposition, but it does not consider the possibility that pesticides in combination may produce toxicological effects via additive or synergistic interactions. Although the combined effects of pesticides within the same class could be predicted based on our understanding of their mechanism of toxic action, effects of mixtures from different classes are more difficult to understand and predict. Pesticide combinations, chemical interactions, doses, and biological responses are parameters which make toxicological study of pesticide mixtures highly complex in methodical analysis (Gennings et al., 2004) (McCarty and Borgert, 2006), yet toxicological evaluation of pesticide mixtures at dietary exposure level is important in terms of human population safety. Although residual pesticide contamination in food is variable, estimation of maximum dietary intake of pesticides tend to be under the ADI level for adults in European Union (Leblanc et al., 2000; Lorenzin, 2007; Nasreddine and Parent-Massin, 2002). Indeed, reduction of residues may occur in storage or from washing, trimming, and processing. Nevertheless, a recent study performed on ready to

eat meals in Italy revealed the presence of a maximum of 10 different pesticides by meal and the estimated daily intake was in a range of 2.6–73% of ADI for adults, 4.9–109% of ADI for teenagers and 9.8–219% of ADI for children's (Lorenzin, 2007).

Liver cancer models are often used to test carcinogenicity of substances. The alternative Solt–Farber model (10-days protocol) induces efficiently hepatocellular carcinomas (HCC) in liver by treatments with diethylnitrosamine (DEN), 2-acetylaminofluorene (2AAF) and 70% partial hepatectomy (PH) to the rats (Marche-Cova et al., 1995; Perez-Carreón et al., 2006) like in the original 4-weeks protocol (Solt, 1976). In early steps, altered hepatocyte foci (AHF) and nodular lesions are induced in a short period (4 weeks). Its enhanced efficacy for nodule induction is based in the two step initiation-promotion theory in which the DEN-initiated cells with a resistant phenotype are able to proliferate under a selective promotion provoked by the 2AAF/PH treatment. The cellular phenotype of AHF and nodule developing using the Solt–Farber protocol has revealed striking similarities in experimental and human hepatocarcinogenesis. Consequently, nodular hepatocyte lesions, preceding HCC, represent the most prevalent form of hepatic preneoplasia observed in both animals and humans (Banasch et al., 2003). The hepatocarcinogenesis model has been used as a medium-term carcinogenic-test system due to accelerated induction of hepatocyte nodules and possibility of quantitative detection of the well characterized tumor markers  $\gamma$ -glutamyl transpeptidase (GGT) and glutathione S-transferase P (GSTP) (Enzmann et al., 1998; Ito et al., 2003).

The 12 pesticides investigated in the present study have been evaluated individually by the different national and international relevant organizations that meet current human health and safety standards, including absence of cancer-initiating activity. Table 1 shows the selected pesticides and describes their chemical class, agronomical use and the carcinogenicity data in rodents, involved in their regulatory approval. Our investigation explores the influence of a mixture of these pesticides in medium-term rat hepatocarcinogenesis. We have tested the possible tumorigenic and cocarcinogenic influence of this combination at ADI level in a set of liver carcinogenesis protocols. Induction of preneoplastic hepatocyte nodules is evaluated on liver through quantitatively determination of enzymatic markers GGT and GSTP.

## 2. Material and methods

### 2.1. Animals and treatment procedures

Male F344 rats weighing 150–190 g were purchased from the institutional production unit of experimental laboratory animals (UPEAL-Cinvestav, Mexico, DF, Mexico). All experiments followed institutional animal care and use committee guidelines. Groups from 5 to 9 rats were treated as indicated in Fig. 1. Treatments with the pesticide mixture or vehicle control were coupled to each of the four carcinogenic following protocols: the complete hepatocarcinogenesis protocol (DEN + 2AAF + PH; Fig. 1A) and three incomplete protocols in which one or both of the inducing (DEN) or tumor promoting (2AAF) agents are absent (Fig. 1B–D). High purity analytical standard of pesticides were obtained from Sigma–Aldrich, France. They were dissolved in DMSO and then suspended in 1% carboxymethyl cellulose (CMC) solution. Suspensions of pesticides are described in Table 2. Concentration of pesticides (carbofuran, chlorpyrifos, diazinon, dicofol, endosulfan, iprodione, mancozeb, maneb and procymidone) was calculated by considering the ADI indicated by the JMPR through 2006 (JMPR, 2006). The final proportion of DMSO in the 1% CMC solution corresponded to 0.3% for P1X and V1X and 1.7% for P10X and V10X. Rats were treated by gavage five times a week during 8 weeks with 0.5 ml of pesticide suspensions (P1X or P10X) or vehicle controls (V1X or V10X) (Fig. 1). DEN-treated rats were injected i.p. with a solution 80 mg/ml of DEN (Sigma–Aldrich, Toluca, Mexico) at a dose of 200 mg/kg. Rats treated with 2AAF received by gavage a suspension 1% of 2AAF in 1% CMC at a dose of 25 mg/kg during three consecutive days as reported elsewhere (Semple-Roberts et al., 1987). All rats were subjected to a 70% partial hepatectomy (PH) (Martins et al., 2008). Animals were sacrificed by exsanguination under ether anesthesia, livers were excised, washed in physiological saline solution, frozen in 2-methyl butane with liquid nitrogen or immersed in RNAlater (Sigma, St. Louis, MO), and stored at  $-80^{\circ}\text{C}$ .

**Table 1**  
Characteristics of selected pesticides and main carcinogenicity data.

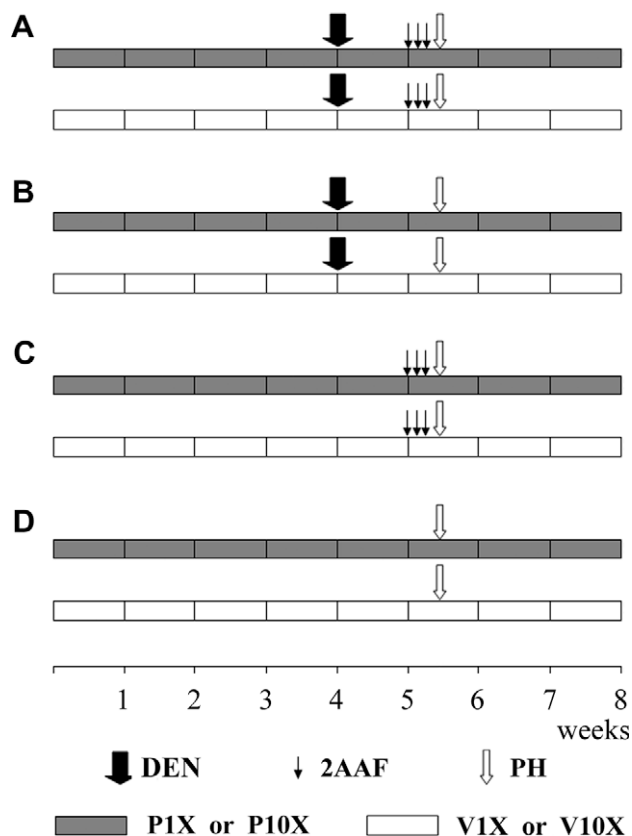
Pesticide ISO-name	CAS no.	Chemical class <sup>a</sup>	Main use <sup>b</sup>	Reports of carcinogenicity tests in rodents	Human carcinogen classification	
					EPAC <sup>c</sup>	IARC <sup>d</sup>
Alachlor	15972-60-8	OC	HB	Nasal neoplasms in rats (Genter et al., 2000)	L (high dose) (C) NL (low dose) (D)	ND
Atrazine	1912-24-9	HC OC	HB	Mammary tumors increased only in female Sprague–Dawley rats (Stevens et al., 1999)	NL (D) (inconclusive)	Group 3, Vol.73
Carbofuran	1563-66-2	CB HC	IN AC	ND	NL (D)	ND
Chlorpyrifos	2921-88-2	OP HC	IN AC	Negative in F344 rats, long term study (Yano et al., 2000)	U (E)	ND
Diazinon	333-41-5	OP HC	IN AC	Lack of induction of preneoplastic liver cell foci (Kato et al., 1995)	NL (D)	ND
Dicofol	115-32-2	OC	AC	Hepatocarcinogen in male B6C3F1 mice (Program, 1978)	PO (C)	Group 3, Vol.30-57
Endosulfan	115-29-7	OC HC	IN AC	Promotes the growth of altered hepatic foci in rats (Fransson-Steen et al., 1992)	NL (D)	ND
Iprodione	36734-19-7	HC	FU	ND	L (C)	ND
Mancozeb	7/1/18	TC	FU	Multipotent carcinogenic agent in rats (Belpoggi et al., 2002)	PR (B2)	ND
Maneb	12427-38-2	TC	FU	Tumor initiating activity in mouse skin (Mehrotra et al., 1987)	PR (B2)	Group 3, Vol. 12-57
Procymidone	32809-16-8	OC HC	FU	Testicular interstitial cell tumors in male rats (Hosokawa et al., 1993)	PR (B2)	ND
Rotenone	83-79-4	HC	IN AC	Negative in female Wistar rats over 50 tissues (Greenman et al., 1993)	U (E)	ND

<sup>a</sup> Class: OC, organochlorine; HC, heterocyclic; CB, carbamate; OP, organophosphorus; TC, thiocarbamate.

<sup>b</sup> Use, HB, herbicide; IN, insecticide; AC, acaricide; miticide; FU, fungicide.

<sup>c</sup> US Environmental Protection Agency (PR, probable (B); PO, possible (C); L, likely (C); U, unlikely (D); NL, not likely (E)).

<sup>d</sup> International Agency for Research on Cancer [Group 3 = not classifiable as to carcinogenicity to humans, ND = not determined].



**Fig. 1.** Hepatocarcinogenic experimental protocols and pesticide treatments. Rats were treated with pesticides (gray) or vehicle (white) during 8 weeks; P1X, pesticide mixture at ADI dose; P10X, pesticide mixture at 10X-ADI dose (see Table 2 for concentrations); V1X, vehicle 0.3% DMSO in 1% CMC; V10X, vehicle 1.7% DMSO in 1% CMC. After week 4 animals were divided in four groups (A–D). A, complete protocol DEN + 2AAF + PH; B, protocol DEN + PH; C, protocol 2AAF + PH; D, protocol PH. DEN, 200 mg/kg, ip; 2AAF, 25 mg/kg oral; PH, 2/3 partial hepatectomy.

## 2.2. Histological analyses of liver GGT<sup>+</sup> lesions

Representative 15  $\mu\text{m}$  thick sections from liver slices were stained for GGT activity, in accordance with Rutenburg et al. (Lee, 1986; Rutenburg et al., 1969). Briefly, histological sections were fixed 10 min with 96% ethanol and then were incubated 30 min with a solution 125  $\mu\text{g}/\text{ml}$  of  $\gamma$ -glutamyl-4-methoxy-2-naphthylamine, 500  $\mu\text{g}/\text{ml}$  of glycylglycine, 500  $\mu\text{g}/\text{ml}$  of fast blue BB salt in 100 mM Tris pH 7.4. Slides were rinsed with deionized water and incubated 5 min with a 0.1 M  $\text{CuSO}_4$  solution, slides were rinsed, dried and scanned at 1200 dpi resolution.

Detection of GGT<sup>+</sup> lesions on liver was performed on an average area of 250  $\text{mm}^2$  from six histological sections for each rat. The image processing software for microscopy WCIF ImageJ (Abramoff, 2004) was used for image analyses. Red col-

or GGT<sup>+</sup> regions were detected using color threshold tools in the software. Hepatocyte GGT<sup>+</sup> foci or nodules were determined by filtering those region of interest with an area > 0.03  $\text{mm}^2$ .

## 2.3. Quantitative RT-PCR determination of hepatic Gstp mRNA level

RNA later-stabilized liver samples were utilized for total RNA isolation by column based extraction (RNeasy Mini kit, Qiagen, France). Quantity and purity were determined by measure DO at 260/280 nm in a UV-spectrophotometer. RNA quality was verified by agarose-gel electrophoresis in which rRNA 28S/18S > 1.7 ratios were obtained.

cDNA synthesis was performed from 2  $\mu\text{g}$  of total RNA using SuperScript II-reverse transcriptase reagents (Invitrogen, Cergy Pontoise, France). Quantitative PCR assay was carried out using TaqMan Gene expression assays in an ABI PRISM 7000 sequence detection system (Applied Biosystem, Courtaboeuf, France). FAM dye-labeled probes (exon-exon boundary) for rat glutathione S-transferase pi (Rn00821792\_g1) and for rat beta-actin (Rn00667869\_m1) gene were purchased from Applied Biosystem. Expression signals were calculated by interpolating in a standard curve made by serial dilutions of cDNA obtained from RNA of a liver tumor positive to GSTP. GSTp data were normalized against beta-actin gene expression.

## 2.4. Western blot analysis of liver GSTP protein level

Frozen liver samples (100 mg/ml) were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1  $\mu\text{g}/\text{ml}$  each of aprotinin, leupeptin, pepstatin A, bestatin A). After protein quantification by a Lowry related method (RC DC Protein Assay, BioRad), samples were prepared in a 5X Laemmli buffer. Proteins (25  $\mu\text{g}$ ) were separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked by incubating for 1 h at room temperature with 5% non-fat milk, 0.1% Tween 20 in tris buffered saline (TBS). Membranes were incubated overnight at 4  $^{\circ}\text{C}$  with a monoclonal anti- $\beta$ -Actin antibody (Sigma, Saint Louis, USA) or a serum antibody to human GSTP which was highly immunoreactive with the rat enzyme. This antibody was raised in rabbit in our laboratory using the human protein as antigen (94% sequence identity to the rat protein). After washings, membranes were incubated during 1 h with an anti-mouse or anti-rabbit peroxidase-conjugated secondary antibody (Sigma). Enhanced chemifluorescent signal (ECL Plus, Amersham Biosciences, Germany) of immune complexes was visualized in a Storm 860 phosphorimager (Amersham Biosciences). The band intensity was measured using ImageJ tools.

## 2.5. Statistical analyses

Data from histological analyses of GGT, GSTP protein and mRNA determinations were expressed as mean  $\pm$  SD of different animals as indicated in figure footnotes. Statistical significant differences between experimental groups were determined by one-way analysis of variance (ANOVA) or by Student's *t*-test. Significance was defined as a *p*-value of less than 0.05.

## 3. Results

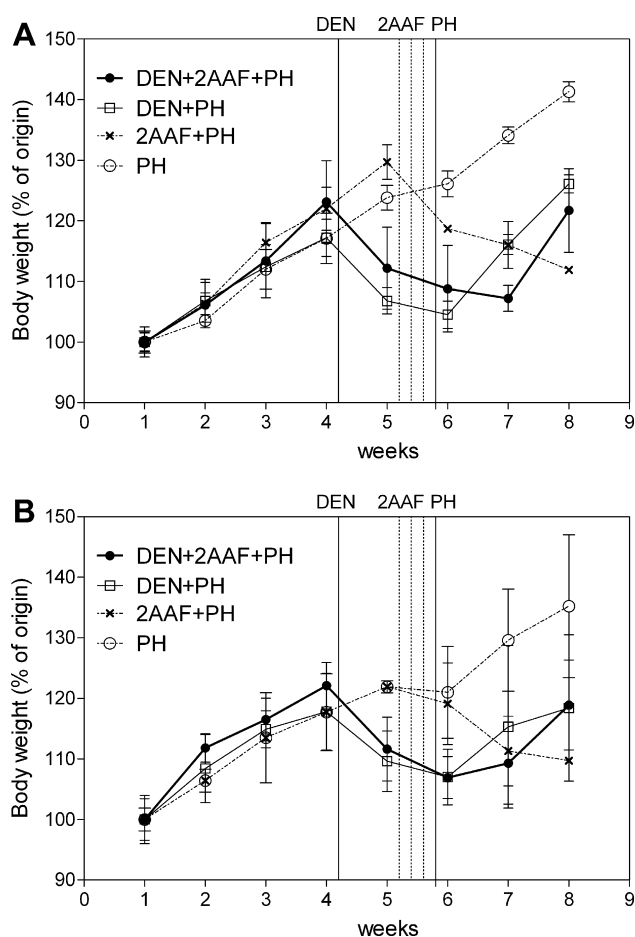
Animals were weighed weekly and body weight (bw) change in vehicle treated rats was found to depend on carcinogenic protocol (Fig. 2A). During the first 4 weeks, all rats gained between 10.2 and 15.2 g/week. From weeks 4 to 5, only DEN-treated rats lost between 8.0% and 8.7% of bw 1 week after carcinogen treatment.

**Table 2**  
Suspensions of pesticides.

Pesticide	<sup>a</sup> ADI (mg/kg/day)	Year of evaluation	PIX		PIOX	
			mg/ml	<sup>b</sup> ADI-folds	mg/ml	<sup>b</sup> ADI-folds
Alachlor	ND		0.0002		0.002	
Atrazine	ND		0.0001		0.001	
Carbofuran	0.002	2002	0.0008	1	0.008	10
Chlorpyrifos	0.01	2004	0.004	1	0.04	10
Diazinon	0.002	1993	0.0008	1	0.008	10
Dicofol	0.002	1992	0.001	1.3	0.01	12.5
Endosulfan	0.006	1998	0.0024	1	0.024	10
Iprodione	0.06	1995	0.024	1	0.12	5
Mancozeb	0.03	1993	0.02	1.6	0.1	8.3
Maneb	0.03	1993	0.02	1.6	0.1	8.3
Procyimidone	0.1	1989	0.04	1	0.2	5
Rotenone	ND		0.05		0.25	

<sup>a</sup> Acceptable daily intake according to (JMPR, 2006).

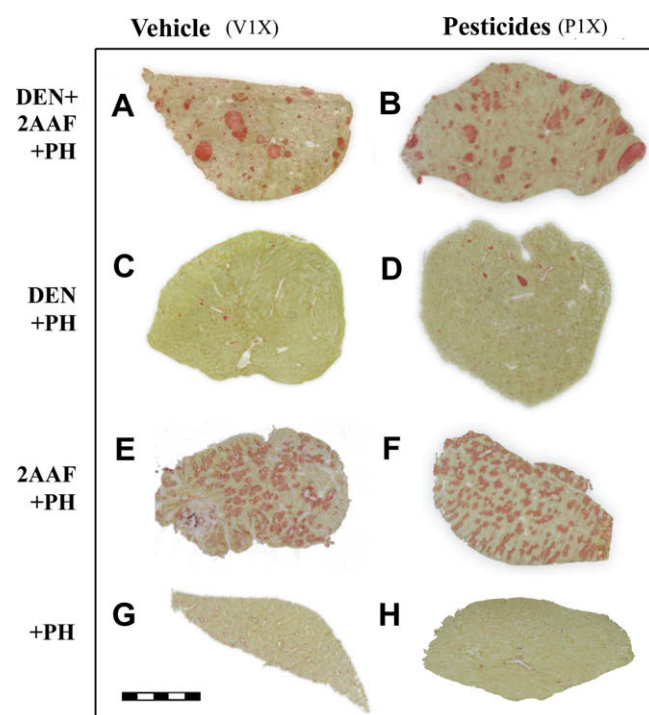
<sup>b</sup> ADI-folds considering 0.5 ml of suspension/day and rat body-weight = 200 g.



**Fig. 2.** Rat body-weight measurement during 8 weeks experimental treatments. A, Vehicle treated rats V1X with each of the four carcinogenic protocols. B, Pesticide treated rats P1X with each of the four carcinogenic protocols. Data were normalized as percentage of initial weight (mean  $\pm$  SD). There was no statistically difference between A and B data by ANOVA.

During liver regeneration phase (weeks 6–8), rats submitted to the following three protocols; DEN + 2AAF + PH, DEN + PH and PH recovered between 5.5% and 27% of bw. In contrast, rats of 2AAF + PH protocol lost between 0.1% and 7.9% of bw. Body weight of rats treated with pesticides showed no statistically significant difference (Fig. 2B) when compared to vehicle treatments. Similar result was found in P10X-treated rats (data not shown). Thus, chemical carcinogens DEN and 2AAF affected body weights of animals in the hepatocarcinogenesis protocols but pesticide treatment not.

Presence of GGT positive nodules and AHF in liver was utilized as biomarker of carcinogenic effects (Fig. 3). As positive control, the DEN and 2AAF carcinogens in the complete protocol produced numerous hepatocyte nodules GGT<sup>+</sup> (Fig. 3A). Addition of the pesticide mixture during 8 weeks at ADI level in the complete protocol did not change induction of GGT positive lesions (Fig. 3B). The DEN + PH protocol produces a few GGT<sup>+</sup> hepatocyte foci (AHF), and no difference between pesticides or vehicle treatment at both doses was seen (Fig. 3C and D; P10X and V10X images are not shown). Histological GGT activity in 2AAF + PH protocol (Fig. 3E and F) was marked in small cells of periportal areas and according to previous reports using similar protocols it corresponds to oval cell proliferation (Petersen et al., 1998; Qin et al., 2004). GGT activity in this protocol did not show a hepatocyte nodular pattern; hence GGT<sup>+</sup> lesions were not quantifiable. In PH protocol (Fig. 3G



**Fig. 3.** Comparison of pesticide effects by histochemical detection of GGT-positive lesions on rat livers. Liver sections (15  $\mu$ m) were stained to detect GGT activity (red color) in pesticide mixture (P1X) or vehicle (V1X) treated rats as indicated. A and B, hepatocyte nodules were induced in protocol DEN + 2AAF + PH. C and D, small GGT positive hepatocyte foci were induced in DEN+PH protocol. E and F, GGT positive oval cell proliferation in portal areas was induced in 2AAF + PH protocol. G and H, basal GGT activity in bile-duct epithelial cell was detected in PH protocol. Bar scale is 5 mm.

and H), GGT expression was minimal in biliary tracts such as those reported in normal livers.

Quantitative data on GGT-hepatocyte lesions is summarized in Table 3. The proportion of GGT<sup>+</sup> altered tissue by the DEN and 2AAF in complete protocol was from 4.12% (V1X) to 5.76% (V10X) and lesion number from 18 (V1X) to 21 (V10X) by cm<sup>2</sup>. The pesticide mixture treatment in the complete protocol (Table 3a) at both doses (P1X and P10X) produced essentially the same results as the vehicle treatments (V1X and V10X). When control treatments with a difference in DMSO amount (V1X, 0.3% and V10X 1.7%) were compared, the proportion, quantity and size of nodules were increased between 17% and 39%, indicating that DMSO might give additional co-carcinogenic effect. The GGT<sup>+</sup> area of hepatocyte lesions in the DEN + PH protocol (Table 3b) was notably decreased (20-fold) when compared to the complete protocol. Treatment with the pesticide mixture at ADI level in the DEN + PH protocol, showed not statistically significant difference in liver GGT<sup>+</sup> lesions when compared to vehicle treatment.

Increasing levels of GSTP protein has been considered an important tumor marker in several types of cancer, including chemical-induced HCC in rats (Pitot et al., 1996; Tew, 2007). The GSTP protein level in liver extracts from treated rats is shown in Fig. 4 (densitometry A and blot image B). Higher level of GSTP protein was induced by DEN and 2AAF in the complete protocol than the incomplete protocols. In pesticides-treated rats, the GSTP marker was found essentially at the same level to those rats treated with vehicle in all protocols. GSTP-level change was evident by type of carcinogenic protocol, the complete protocol induced more GSTP >5-fold than protocol DEN + PH, >3-fold than 2AAF + PH and >10-fold than PH protocols (Fig. 4A).

Gstp mRNA level in experimental groups was determined by quantitative RT-PCR as a more sensitive detection method

**Table 3a**  
Quantitative data for GGT<sup>+</sup> hepatocyte lesions in treated rats. Protocol: DEN + 2AAF + PH.

Treatment	Rats	Proportion of GGT <sup>+</sup> tissue (%)		Nodule quantity (Number/cm <sup>2</sup> )		Nodule area			
						Average (mm <sup>2</sup> )		Largest (mm <sup>2</sup> )	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
V1X	8	4.12	3.95	18.00	5.33	0.21	0.05	0.91	0.20
P1X	8	4.56	3.73	18.86	6.30	0.22	0.05	0.99	0.20
V10X	8	5.76	4.72	21.04	11.7	0.26	0.05	1.23	0.20
P10X	5	5.16	2.46	21.44	5.86	0.24	0.04	1.57	0.30

No significant difference was found between V and P experimental groups.

\* Statistical difference ( $p < 0.05$ ) to V1X by Student's test.

**Table 3b**  
Quantitative data for GGT<sup>+</sup> hepatocyte lesions in treated rats. Protocol: DEN + PH.

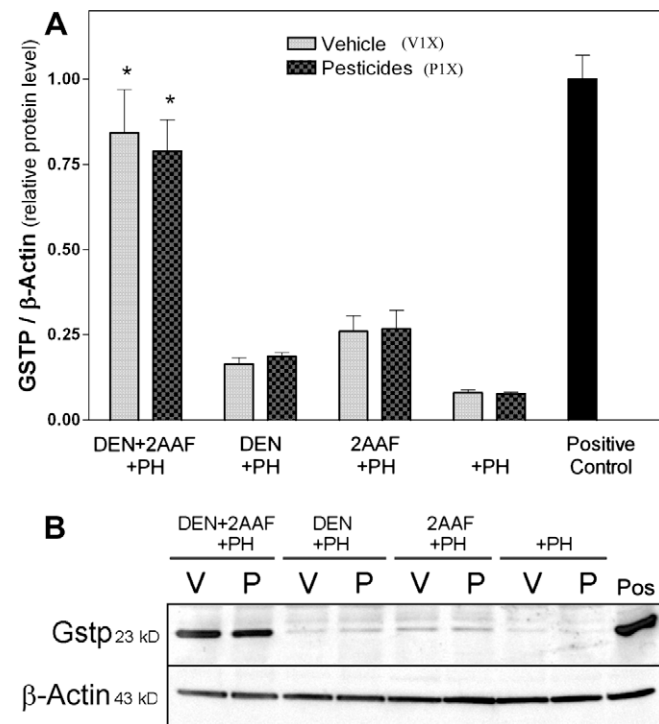
Treatment	Rats	Proportion of GGT <sup>+</sup> tissue (%)		AHF quantity (Number/cm <sup>2</sup> )		AHF area			
						Average (mm <sup>2</sup> )		Largest (mm <sup>2</sup> )	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
V1X	7	0.20	0.12	2.63	1.92	0.09	0.01	0.13	0.02
P1X	8	0.24	0.13	3.72	2.19	0.07	0.01	0.11	0.03

No significant difference was found between V and P experimental groups.

AHF = alerted hepatocyte foci.

(Fig. 5). Treatment with the carcinogens DEN and 2AAF in complete protocol showed higher *Gstp* expression (17-fold) than the incomplete protocol DEN + PH or 27-fold more than 2AAF+PH protocol and 250-fold more than PH protocol. Consistent with GSTP protein

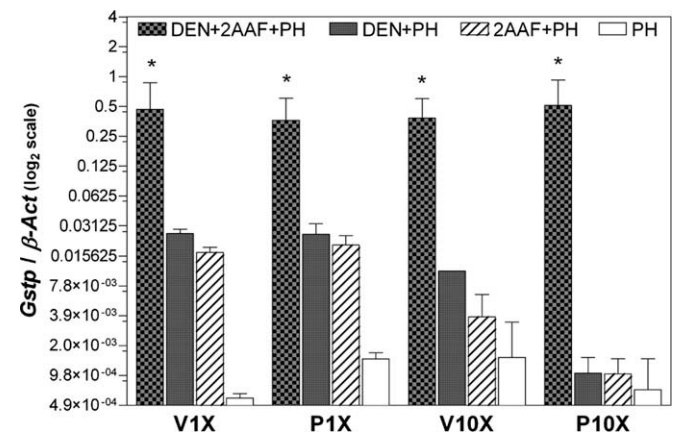
detection, the mRNA levels in pesticide-treatments at P1X and P10X doses were similar or lower than the corresponding vehicle treatments. Thus, pesticide mixture treatment at ADI level was unable to enhance or substitute the initiating or promoting effects of the known carcinogens DEN or 2AAF in liver.



**Fig. 4.** GSTP protein level on liver of rats treated with pesticides or vehicle. A, GSTP was detected by western blot on liver extracts of rats submitted to carcinogenic protocols. The blot intensity of GSTP was normalized with  $\beta$ -actin and expressed as mean  $\pm$  SD of four rats analyzed independently. B, representative blot image from pools of four liver extracts of rats treated with P1X or V1X. V = Vehicle; P = Pesticides; Pos, positive control obtained from a HCC induced on rat. Significant differences from non-complete hepatocarcinogenesis protocols ( $p < 0.001$ ; ANOVA Bonferroni's Multiple Comparison Test). Means of comparison V vs. P were not statistically significant in all type of protocols.

#### 4. Discussion

In this report, we studied the possible synergism of a pesticide mixture at ADI and 10-fold ADI levels on liver carcinogenesis, using alternative approaches of the Solt and Farber protocol. The carcinogenic parameters were histological quantitative data of GGT<sup>+</sup> nodules or altered hepatocyte foci, and GSTP expression at mRNA and protein levels on liver extracts. Pesticide-treated rats (P1X and P10X, respectively) had the same number of altered hepatocyte lesions and the same GSTP expression as control rats,



**Fig. 5.** Effects of pesticides and vehicle on GSTP mRNA levels in rat livers. Quantitative RT-PCR experiment in two steps was performed from 2  $\mu$ g of total RNA and specific Taqman probes to detect *Gstp* or  $\beta$ -Act genes. Data are presented as mean of normalized ratio  $\pm$ SD of three animals by each experimental group. Significant differences from non-complete hepatocarcinogenesis protocols ( $p < 0.001$ ; ANOVA Bonferroni's Multiple Comparison Test). Means difference of comparisons V1X vs. P1X and V10X vs. P10X independently of protocol were not statistically significant.

whatever the carcinogenesis protocol. On this basis, we concluded that the selected pesticide mixture is deprived of additive/synergistic effects on tumorigenesis at comparative low doses tested in a medium-term hepatocarcinogenesis model.

The potential for toxicological additivity of pesticides in combination is undetermined and it is complicated to estimate it without experimentation. The lack of carcinogenic influence of the mixture tested in this study may be separated on three aspects: (a) The lack of co-carcinogenic effect signifies the incapability of the pesticide mixture to enhance the effect of DEN and 2AAF as carcinogens. (b) The expected lack of cancer-initiating activity implying an absence of effect in induction of genetically altered cell populations by the mixture. (c) The lack of tumor promoting activity of the mixture which represents the incapacity to promote the selective cellular proliferation of carcinogen-initiated cells.

From the twelve pesticides in the mixture, ten have been previously evaluated in rodent carcinogenicity studies. With exception of chlorpyrifos, diazinon and rotenone, the other pesticides have been reported with individual positive tumorigenic potential (Table 1), alachlor in respiratory epithelial cells (Genter et al., 2000), atrazine in mammary gland (Stevens et al., 1999), dicofol, endosulfan and mancozeb in liver (Belpoggi et al., 2002; Fransson-Steen et al., 1992; Program, 1978), maneb in skin (Mehrotra et al., 1987) and procymidone in testis (Greenman et al., 1993). In addition, these previous studies report rather high doses of pesticides given to the animals. For example, altered hepatic foci in rats were produced with 300 ppm of endosulfan (Fransson-Steen et al., 1992), this dose could be estimated to >4000-fold ADI level if a rat (250 g) eat 20 g of food by day. In the same way, rats treated with 1000 ppm of mancozeb in food developed HCC in a long term period (Belpoggi et al., 2002), this dose could be estimated to >2500-fold ADI level. High dose studies of chemicals could offer more probabilities to find toxicological effects and to provide reference doses with adverse significance (Ames and Gold, 1997). However, in regulatory evaluation of non-genotoxic chemicals such as pesticides, possible cancer risk at high doses (exceeding a threshold) would not be considered as relevant at low doses. So, when human exposition to such chemicals is well defined at residual levels and involving combinations, extrapolation from studies is complicated without experimentation in models.

In a number of countries, the highest amount of most of pesticide residues detected in food has been estimated to be under ADI levels for adults. For example I was <4% of ADI in France (Leblanc et al., 2000), <73% in Italy (Lorenzin, 2007), <10% in the European Union (Nasreddine and Parent-Massin, 2002), <80% in Korea (Chun and Kang, 2003) and ranging from 2% to 180% of ADI for fenitrothion in Brazil (Caldas and Souza, 2004). In our study, in order to take into consideration realistic human exposure levels, we derived the dose administered to the animals from the ADI even though this value is at least one hundred fold lower than the NOAEL. The 10-fold ADI dose was used as an excess treatment.

Although there is a public concern about cancer risk of pesticide mixtures, there are few carcinogenicity-related studies at low dose with respect to the large variety of pesticides in current use. Rat liver carcinogenesis test is often used, because of the great hepatic potential of this animal for metabolic activation of xenobiotics. A well-established similar carcinogenesis test is the medium-term bioassay of Ito and co-workers, focused on the evaluation of promoting activity of various substances (Ito et al., 2003). Their protocol combined DEN treatment with PH and used the histological presence of GSTP positive hepatocyte foci as carcinogenic marker. Using this bioassay, they tested the promoting activity of 19 organophosphorus plus one organochlorine pesticides in combination at ADI levels. Results were negative (Ito et al., 1996). These data, although examining a different pesticide mixture, are in agreement with our evaluation at the ADI level.

Different possibilities were evaluated within the known DEN–2AAF–PH carcinogenesis model (Fig. 1). Independently of pesticide or vehicle treatment, the four alternative carcinogenic protocols performed in this study produced different cellular and tumor marker responses. The complete protocol was able to induce numerous hepatocyte nodules as reported in the original Solt and Farber protocol (Semple-Roberts et al., 1987). In contrast, the absence of one on the two carcinogens (incomplete protocols) affected nodule induction. Livers of the DEN + PH protocol showed few and small altered hepatocyte foci. Singularly, the 2AAF + PH protocol formed periportal GGT<sup>+</sup> areas that could correspond to oval cell proliferation. GGT expression in oval cells has been mentioned in protocols in which hepatocyte proliferation is inhibited by selective carcinogens such as 2AAF (Petersen et al., 1998). Furthermore, the 2AAF + PH protocol was more toxic to the rats than the other protocols during the liver regeneration period with an increase in rat mortality (data not shown) and a continuous loss of body weight after PH (Fig. 2).

Histological presence of GGT or GSTP positive hepatocyte foci usually represents an end point marker for several xenobiotics carcinogenicity (Ito et al., 2003; Pitot et al., 1991, 1996). However, chemicals such as proliferators of peroxisome induce GSTP-negative foci in rodents that must be identified with other markers. Proliferation of peroxisome occurring in rodents experiments is not considered as a risk factor for humans (Lai, 2004). Even though the possibility that pesticides act as proliferators of peroxisome was not explored in this report, our histological analyses by hematoxylin and eosin staining in pesticide treated rats and PH protocol showed normal appearance of liver without hepatic foci (data not shown). With or without pesticides GSTP expression was detected in liver extracts at protein and mRNA level in the four types of protocols evaluated. Moreover, quantitative RT-PCR detection of GSTP would be a sensitive alternative method of the classical histological detection used as an end point carcinogenesis marker.

Epidemiological and experimental studies showed that the major risk factors for cancer are tobacco smoke, chronic diseases produced by infections, dietary imbalances and hormonal disorders (Swirsky Gold et al., 1997). However, worrying about the presence of pesticide residues in the diet, a majority of consumers have got the erroneous conviction that such an exposure could represent a serious cancer hazard (Ames and Gold, 1997; Gold et al., 2001). Indeed, also being at variance with consumer feelings, a literature review covering 14 yr from 1985 in toxicology and human risk concluded that exposure to mixtures of pesticides at low doses does not represent a potential source of concern to human health (Carpay et al., 2000). Nevertheless, occurrence of a high cancer incidence worldwide coupled to an accumulative environmental pollution give reasons for a continuous toxicological testing of xenobiotics to define how substance combinations could be adverse to human health. Considering difficulty to extrapolate toxicological data from rat models to humans, our results showing the lack of tumor promoting and co-carcinogenic effect of a representative pesticide mixture, contribute to the assertive concept that the ADI corresponds to a safe intake level to humans even in chronic simultaneous multiple substances exposures.

#### Conflict of interest statement

The authors declare that there are no conflicts of interest.

#### Acknowledgements

We are grateful to F. Blas-y-Estrada and R. Gazel for technical support at the animal facility in France and J. Fernandez, M. Flores,

R. Leyva, and R. Gaxiola for technical support at the animal facility in Mexico.

## References

- Abramoff, M.D., Magelhaes, P.J., Ram, S.J., 2004. Image processing with ImageJ. *Biophoton. Int.* 11, 36–42.
- Ames, B., Gold, L.S., 1997. Environmental pollution, pesticides, and the prevention of cancer: misconceptions. *FASEB J.* 11, 1041–1052.
- Bannasch, P. et al., 2003. Significance of hepatic preneoplasia in risk identification and early detection of neoplasia. *Toxicol. Pathol.* 31, 134–139.
- Belpoggi, F. et al., 2002. Results of long-term experimental studies on the carcinogenicity of ethylene-bis-dithiocarbamate (Mancozeb) in rats. *Ann. NY Acad. Sci.* 982, 123–136.
- Caldas, E.D., Souza, L.C., 2004. Chronic dietary risk for pesticide residues in food in Brazil: an update. *Food Addit. Contam.* 21, 1057–1064.
- Carpay, S.A. et al., 2000. Health risk of low-dose pesticides mixtures: a review of the 1985–1998 literature on combination toxicology and health risk assessment. *J. Toxicol. Environ. Health B Crit. Rev.* 3, 1–25.
- Chun, O.K., Kang, H.G., 2003. Estimation of risks of pesticide exposure, by food intake, to Koreans. *Food Chem. Toxicol.* 41, 1063–1076.
- Enzmann, H. et al., 1998. Short- and intermediate-term carcinogenicity testing – a review. Part 1: the prototypes mouse skin tumour assay and rat liver focus assay. *Food Chem. Toxicol.* 36, 979–995.
- Fransson-Steen, R. et al., 1992. The insecticide endosulfan and its two stereoisomers promote the growth of altered hepatic foci in rats. *Carcinogenesis* 13, 2299–2303.
- Gennings, C. et al., 2004. A novel flexible approach for evaluating fixed ratio mixtures of full and partial agonists. *Toxicol. Sci.* 80, 134–150.
- Genter, M.B. et al., 2000. Evolution of alachlor-induced nasal neoplasms in the Long-Evans rat. *Toxicol. Pathol.* 28, 770–781.
- Gold, L.S., Slone, T.H., Ames, B.N., Manley, N.B., 2001. Pesticide residues in food and cancer risk: a critical analysis. In: Krieger, R. (Ed.), *Handbook of Pesticide Toxicology*, second ed. Academic Press, San Diego, CA, pp. 799–843 (Chapter 38).
- Greenman, D.L. et al., 1993. Bioassay for carcinogenicity of rotenone in female Wistar rats. *Fundam. Appl. Toxicol.* 20, 383–390.
- Herrman, J.L., Younes, M., 1999. Background to the ADI/TDI/PTWI. *Regul. Toxicol. Pharmacol.* 30, S109–S113.
- Hodgson, E., Levi, P.E., 1996. Pesticides: an important but underused model for the environmental health sciences. *Environ. Health Perspect.* 104 (Suppl. 1), 97–106.
- Hosokawa, S. et al., 1993. Effects of procymidone on reproductive organs and serum gonadotropins in male rats. *J. Toxicol. Sci.* 18, 111–124.
- Ito, N. et al., 1996. Effects of pesticide mixtures at the acceptable daily intake levels on rat carcinogenesis. *Food Chem. Toxicol.* 34, 1091–1096.
- Ito, N. et al., 2003. A medium-term rat liver bioassay for rapid in vivo detection of carcinogenic potential of chemicals. *Cancer Sci.* 94, 3–8.
- JMPR, J.F.W.M.o.P.R., 2006. Inventory of IPCS and other WHO pesticide evaluations and summary of toxicological evaluations performed by the Joint Meeting on Pesticide Residues (JMPR) through 2006. In: *International Programme on Chemical Safety*, pp. 1–53.
- Kato, T. et al., 1995. Lack of promoting activity of four pesticides on induction of preneoplastic liver cell foci in rats. *Teratog. Carcinog. Mutagen.* 15, 251–257.
- Lai, D.Y., 2004. Rodent carcinogenicity of peroxisome proliferators and issues on human relevance. *J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev.* 22, 37–55.
- Leblanc, J.C. et al., 2000. Estimation of the dietary intake of pesticide residues lead cadmium arsenic and radionuclides in France. *Food Addit. Contam.* 17, 925–932.
- Lee, B.C., 1986. Concerning materials for gamma-glutamyl transpeptidase histochemistry. *J. Histochem. Cytochem.* 34, 1243.
- Lorenzin, M., 2007. Pesticide residues in Italian Ready-Meals and dietary intake estimation. *J. Environ. Sci. Health B* 42, 823–833.
- Marche-Cova, A. et al., 1995. Follow-up of GST-P during hepatocarcinogenesis with DEN-2AAF in F344 rats. *Arch. Med. Res.* 26, S169–S173.
- Martins, P.N. et al., 2008. Rodent models of partial hepatectomies. *Liver Int.* 28, 3–11.
- McCarty, L.S., Borgert, C.J., 2006. Review of the toxicity of chemical mixtures containing at least one organochlorine. *Regul. Toxicol. Pharmacol.* 45, 104–118.
- Mehrotra, N.K. et al., 1987. Tumour initiating activity of mancozeb – a carbamate fungicide in mouse skin. *Cancer Lett.* 36, 283–287.
- Nasreddine, L., Parent-Massin, D., 2002. Food contamination by metals and pesticides in the European Union. Should we worry? *Toxicol. Lett.* 127, 29–41.
- Perez-Carreón, J.I. et al., 2006. Gene expression profile related to the progression of preneoplastic nodules toward hepatocellular carcinoma in rats. *Neoplasia* 8, 373–383.
- Petersen, B.E. et al., 1998. Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. *Hepatology* 27, 433–445.
- Pitot, H.C. et al., 1991. Biochemical markers associated with the stages of promotion and progression during hepatocarcinogenesis in the rat. *Environ. Health Perspect.* 93, 181–189.
- Pitot, H.C. et al., 1996. Quantitation of multistage carcinogenesis in rat liver. *Toxicol. Pathol.* 24, 119–128.
- Program, N.T., 1978. National toxicology program. Bioassay of dicofol for possible carcinogenicity. *Natl. Cancer Inst. Carcinog. Tech. Rep. Ser.* 90, 1–99.
- Qin, A.L. et al., 2004. Characterization and enrichment of hepatic progenitor cells in adult rat liver. *World J. Gastroenterol.* 10, 1480–1486.
- Rutenburg, A.M. et al., 1969. Histochemical and ultrastructural demonstration of gamma-glutamyl transpeptidase activity. *J. Histochem. Cytochem.* 17, 517–526.
- Simple-Roberts, E. et al., 1987. Alternative methods of selecting rat hepatocellular nodules resistant to 2-acetylaminofluorene. *Int. J. Cancer.* 40, 643–645.
- Solt, D.a.F., Emmanuel, 1976. New principle for the analysis of chemical carcinogenesis. *Nature* 263, 701–703.
- Stevens, J.T. et al., 1999. A risk characterization for atrazine: oncogenicity profile. *J. Toxicol. Environ. Health A* 56, 69–109.
- Swirsky Gold, L. et al., 1997. Pesticide residues in food: investigation of disparities in cancer risk estimates. *Cancer Lett.* 117, 195–207.
- Tew, K.D., 2007. Redox in redux: emergent roles for glutathione S-transferase P (GSTP) in regulation of cell signaling and S-glutathionylation. *Biochem. Pharmacol.* 73, 1257–1269.
- Yano, B.L. et al., 2000. Lack of carcinogenicity of chlorpyrifos insecticide in a high-dose, 2-year dietary toxicity study in Fischer 344 rats. *Toxicol. Sci.* 53, 135–144.