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Testosterone hydroxylase as multibiomarker of effect in evaluating vinclozolin cocarcinogenesis

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In this work the modulation of the regio- and stereo-selective hydroxylation of testosterone by vinclozolin was studied in evaluating cocarcinogenic properties. Changes of cytochrome P450-(CYP)-catalysed drug metabolism was investigated in liver, kidney and lung microsomes of Swiss Albino CD1 mice of both sexes after single (625 or 1250 mg kg^{-1} b.w.) or repeated (daily 750 mg kg^{-1} b.w. for 3 days) i.p. administrations. Treatment of mice with a single dose of vinclozolin caused in a dose-dependent fashion from 2·1 to 14·1-fold increase in the 7α-, 6β- and 2β-hydroxylations of testosterone in liver. Lower increase in extrahepatic tissues ranging from 2·3 to 8·1-fold for testosterone 6β-, 16β-, 2α- and 2β-hydroxylase activity in the kidney or from 2·2 to 5·1-fold for 6β-, 16α-, 16β- and 2β-hydroxylase activity in the lung were observed. Repeated treatment with this fungicide did not substantially modify the extent and pattern of induction, the liver being the only tissue responsive (up to 7·6-fold increase, 7β-hydroxylation) in both male and female. In the kidney (7α-, 6β-, 16β-, 2α-, 7α-hydroxylations) and lung (6α-, 7α-, 6β-, 16α-, 16β- and 2α-hydroxylations), a typical sex-dependent induction (up to 9·0-fold, 16α-hydroxylation in the lung, female) was achieved. In general, however, vinclozolin has a complex pattern of induction and suppression of CYP-dependent enzymes, as exemplified from the reduced expression of some hydroxylations depending upon dose, sex and organ considered. For example, after a single administration, 16β-hydroxylation was suppressed in liver (up to 78% loss in male, higher dose), whereas 16α-hydroxylation was reduced in kidney up to ~50% in both sexes (at the higher dose). Glutathione S-transferase activity, measured as index of post-oxidative reactions, was markedly increased by vinclozolin in the liver (up to 5·2-fold, female) and kidney (up to 3·9-fold, female) but not in the lung. Because both phase I and phase II reactions were enhanced by vinclozolin treatment in liver and kidney, the ratio between activation/detoxification mechanisms was slightly affected. Conversely, this ratio was shifted toward activating mechanisms in the lung, sustaining, in part, the expression of certain type of tumours tissue-dependent. Taken together, these findings seem to indicate the cotoxic, cocarcinogenic and promoting potential of this fungicide.

Keywords: vinclozolin, testosterone hydroxylase, glutathione S-transferase, cocarcinogenesis.

Introduction

Because large amounts of pesticides are daily released into the environment, they may represent a potential hazard to humans. Most of them, however, are poor initiating agents and epigenetic mechanisms may thus be relevant particularly after long-term exposure at low doses. It has, indeed, been recently suggested that tumorigenesis could be also mediated by non-genotoxic mechanisms such as

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comutagenesis/cocarcinogenesis and promotion (Butterworth 1990, Paolini *et al.* 1994).

Vinclozolin – (*R S*)-3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2, 4-dione – is a selective contact fungicide mainly used on stone fruits, strawberries, vegetables and vines (figure 1). It has been reported that the 2,4-oxazolidinedione ring of the chemical can undergo hydrolysis leading, reversibly, to the formation of 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and, irreversibly, to 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) (Clark 1983, Szeto *et al.* 1989a,b,c). Because not only the parent compound but also these two metabolites were found within the soil, plants, and animals exposed to vinclozolin, M1 and M2 can have a role in assessing potential health risks (Hawkins *et al.* 1990). Mutagenesis studies on vinclozolin show a general tendency to give negative results in the majority of the tests (Ames test, Chinese hamster sister chromatid exchange study, host mediated assay with *Salmonella typhimurium* and dominant lethal assay) (EPA 1985). Positive evidence in *S. typhimurium*, *Schizosaccaromyces pombe* and diploid colonies of *Aspergillus nidulans* (mitotic recombination and mitotic non-disjunction) have been shown in the open literature (Georgopoulos *et al.* 1979, Chiesara *et al.* 1982, Vallini *et al.* 1983).

On the contrary, it was also found to be an inducing agent in the mouse bone marrow micronucleus assay (Hrelia *et al.* 1996). Although vinclozolin has been reported to be non-carcinogenic by FAO/WHO (1986), questionable results have been obtained in the mouse in which it causes leukaemia/lymphoma type tumours in male animals, but the historical controls equalled the treated levels. Some adenoma in the lung of female mice, where the historical control incidence [which varied from 5·6 % (1974–78) to 25·5 % (1978–80)] was significantly lower than that observed in the treated groups, were also recorded (EPA 1985).

The above considerations and the fact that outstanding problems faced in the prediction of potential carcinogens are mainly toxicological, non-genetic (Ashby 1994), prompted us to investigate some toxicological effects at metabolic level as a possible surrogate for cocarcinogenic and promotional activity (Kitchin *et al.* 1993; Paolini *et al.* 1994). The aim of this work was, therefore, to study the ability of vinclozolin to induce CYP changes which are associated to cocarcinogenic properties. For this purpose, the effect of the fungicide on CYP apparatus in murine liver, kidney and lung microsome using the regio- and stereo-selective hydroxylation of testosterone as multibiomarkers of effect was explored. Glutathione S-transferase activity was also measured in order to consider the possible role of the ratio between activating/detoxifying mechanisms in the expression of toxicity in different tissues.

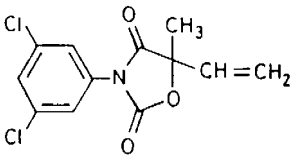

Figure 1. Chemical structure of vinclozolin.

Materials and methods

Chemicals

$NADP^+$, 16α-hydroxytestosterone (16α-TH), corticosterone, testosterone and 4-androsten-3,17-dione (17-TH) were purchased from Sigma Chemical Co. (St Louis, MO, USA); glucose 6-phosphate and glucose 6-phosphate dehydrogenase from Boehringer-Mannheim (Germany), HPLC grade methanol, tetrahydrofurane and dichloromethane from BDH (Poole, UK); 7α-, 6β- and 16β-hydroxytestosterone from Steraloids (Wilton, N.H.); 6α-, 2α- and 2β-hydroxytestosterone were a generous gift from Dr P.G. Gervasi (CNR Pisa, Italy); vinclozolin [(*R S*)-3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione] chemical purity 99·6%, was purchased from Lab Service (Bologna, Italy); all other chemicals and solvents were of highest commercially available purity.

Animal treatment and preparation of subcellular fractions

Male and female Swiss Albino CD1 mice (Nossan, Correzzana, Milan), weighing 28–30 g, were housed under controlled conditions (12 h light–dark cycle, 22 °C, 60 % humidity). They were fed a rodent chow standard alimentation and had tap water *ad libitum*. Vinclozolin was dissolved in corn oil and administered (i.p.) in a single (625 and 1250 mg kg^{-1}) or repeated (daily 750 mg kg^{-1}, for three consecutive days) dose. Six animals for each treatment were employed using different doses corresponding to 50 % and 25 % of LD_{50} which was previously determined using six animals for each dose, eight doses, and calculated using the Litchfield-Wilcoxon method (data not shown). These high doses were selected in order to determine the ability of vinclozolin to affect CYP machinery in terms of 'potentiality', independently from its possible action in human where the exposure is very different either in quantitative terms or time. Controls received vehicle only, under the same conditions. Mice were fasted 16 h prior to death and killed humanely in accordance with approved Home Office procedures appropriate to the species. Liver, kidney and lung were homogenized in 4 vol. (w/v) of 0·05 M Tris–HCl buffer (pH 7·4) with a Teflon pestle (Potter-Elvehjem) and centrifuged at 9000 × *g* for 20 min. The post-mitochondrial supernatant obtained, was centrifuged for 60 min at 105 000 × *g*. The pellet was resuspended in 0·1 M $K_3P_2O_7$ (pH 7·4), 1 mM EDTA and 20 nM BHT, and centrifuged again for 60 min at 105 000 × *g* to obtain the final microsomal fraction. The resultant washed microsomes were then resuspended with a hand-driven homogenizer in 10 mM Tris–HCL buffer (pH 7·4) containing 1 mM EDTA and 20 % glycerol (v/v). Subcellular preparations were immediately frozen in liquid nitrogen, stored at –80 °C and used within a week.

Testosterone hydroxylase activity

Incubation and isolation: Incubations contained microsomes (equivalent to 1–2 mg protein), 0·6 mM $NADP^+$, 8 mM glucose 6-phosphate, 1·4 U glucose 6-phosphate dehydrogenase and 1 mM $MgCl_2$, in a final volume of 2 ml 0·1 M phosphate Na^+/K^+ buffer (pH 7·4). The mixture was preincubated for 5 min at 37 °C. The reaction was performed at 37 °C by shaking and started by the addition of 80 mM testosterone (dissolved in methanol). After 10 min, the reaction was stopped with 5 ml ice-cold dichloromethane and 12 nmol corticosterone (internal standard) in methanol. After 1 min vortexing, phases were separated by centrifugation at 2000 × *g* for 10 min and the aqueous phase was extracted once more with 2 ml dichloromethane. The organic phase was extracted with 2 ml 0·02 N NaOH to remove lipid constituents, dried over anhydrous sodium sulphate and transferred to a small tube. Dichloromethane was evaporated at 37 °C under nitrogen and the dried samples stored at –20 °C. The samples were dissolved in 100 µl methanol and analysed by high performance liquid chromatography (Platt *et al.* 1989).

HPLC separation and quantification: Chromatographic separations were performed using a system consisting of a high-pressure pump (Waters Model 600E, Multisolvent Delivery System), a sample injection valve (Rheodyne Model 7121, Cotati, CA, USA) with a 20 µl sample loop and an ultraviolet detector (254 nm, Waters Model 486, Tunable Absorbance Detector) connected to an integrator (Millennium 2010, Chromatography Manager). For reversed-phase separation of metabolites, NOVA-PACK C18 analytical column (60 Å, 4mm, 3·9 × 150 mm, Waters) was used as stationary phase. The mobile phase consisted of a mixture of solvent A [7·5 % (v/v) tetrahydrofuran in water] and solvent B [7·5 % (v/v) tetrahydrofuran and 60 % (v/v) methanol in water] at a 1 ml min^{-1} flow rate. Metabolite separation was performed by a gradient from 30 % to 100 % (v/v) of solvent B over 30 min. The eluent was monitored at 254 nm and the area under the absorption band was integrated. The concentration of metabolites was determined by the ratio between respective metabolite peak areas and corticosterone (internal standard) and the calibration curves obtained with synthetic testosterone derivatives (Van der Hoeven 1984, Paolini *et al.* 1996a).

Glutatione S-transferase activity: This post-oxidative (microsomal) enzymatic reaction was measured kinetically at 25 °C (pH 6·5) in the presence 1-chloro-2,4-dinitrobenzene as substrate (Habig *et al.* 1974). Incubation was performed in 0·1 M Na^+/K^+ phosphate buffer containing 2·5 nM glutathione. Reaction rates were calculated using the molar extinction coefficient of 13·6 l/(m

Protein concentrations: Protein was determined according to the method described by Lowry *et al.* (1951) and Bailey (1967), using bovine serum albumin as standard. Samples were diluted 200 times to provide a suitable protein concentration.

Statistics and computer analysis

Statistical analysis on biochemical data was performed using Wilcoxon's rank method as reported by Box and Hunter (1978). The software used was Sigma Plot 5·0, Millennium 1·1 and Windows 3·1 run on an AT 486 IBM-compatible computer.

Results

Expression of testosterone hydroxylases in murine liver, kidney and lung after single administration of vinclozolin

No significant differences of either absolute or relative weights in the considered organs were achieved (data not reported).

Table 1 shows the expression of testosterone hydroxylase activity in liver microsomes. We discuss only the most significant changes. Testosterone 6α-hydroxylase activity was induced at both doses from ~2 to 2·2-fold in female mice, whereas 7α-hydroxylase activity was significantly increased ($p<0·01$) from 3·0 to 3·8-fold (in male and female, respectively) at lower dose and from 5·2 to 14·0-fold (in

Table 1. Expression of testosterone hydroxylase in liver microsomes of mice receiving a single dose of vinclozolin.

	Vehicle only (corn oil)		Vinclozolin (625 mg^{-1} kg b.w.)		Vinclozolin (1250 mg^{-1} kg b.w.)	
Metabolites	M	F	M	F	M	F
6α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	87·53± 6·39	83·14± 7·86	146·57± 12·36**	164·25± 13·22**	133·92 6·39**	179·52± 10·51**
7α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	8·10± 0·75	21·38± 2·72	24·34± 2·78**	81·25± 7·61**	113·96± 8·45**	110·89± 9·03**
6β-Hydroxytestosterone (nmol mg^{-1} min^{-1})	0·62± 0·05	0·90± 0·04	1·34± 0·06*	2·27± 0·31**	2·28± 0·03**	3·20± 0·27**
16α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	18·21± 1·15	19·40± 1·42	10·35± 0·92**	22·33± 1·96*	16·57± 1·04	23·59± 2·21*
16β-Hydroxytestosterone (pmol mg^{-1} min^{-1})	88·86± 9·62	81·25± 8·37	64·76± 5·37	135·42± 12·38	19·48± 2·42	59·50± 6·01
2α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	3·37± 0·29	4·08± 0·03	5·65± 0·63**	3·04± 0·26**	5·39± 0·56**	3·76± 0·42
2β-Hydroxytestosterone (pmol mg^{-1} min^{-1})	63·79± 4·77	67·58± 5·73	171·95± 16·75**	267·58± 24·08**	310·19± 20·93**	299·62± 25·32**
4-Androsten-3,17-dione (nmol mg^{-1} min^{-1})	0·93± 0·08	1·34± 0·11	0·72± 0·06	1·32± 0·12	0·54± 0·04	1·33± 0·14

Each value represents the mean ± SD of six independent experiments.

See Materials and Methods section for details and experimental procedures.

(*$p<0·05$, **$p<0·01$) Significant differences between treated groups and their respective controls, using the Wilcoxon's rank method.

female and male, respectively) at higher dose. Testosterone 6β-hydroxylase activity (CYP 3A-like) was enhanced from ~2·2 to ~3·7-fold (averaged between male and female). Vinclozolin was able to significantly ($p<0·01$) increase 2β-hydroxylation at either 625 (from 2·7 to 4·0-fold, in male and in female, respectively) or 1250 mg kg^{-1} b.w. dose (from 4·4 to 4·9-fold, in female and male, respectively). Some inactivating effects were also observed, such as a marked reduction in 16β-hydroxylation (up to 78 %) and 4-androsten-3,17-dione-dependent monooxygenases (up to ~50 %) in male animals at higher dose.

In the kidney, vinclozolin was able to increase the CYP 3A-associated hydroxylation up to 4·6-fold in female at the higher tested dose (table 2). Testosterone 16β-hydroxylase activity was significantly enhanced from ~2·3 to 3·3-fold (in male and female, respectively) either at both doses. 2α-Hydroxylation was, instead, induced in a dose-dependent manner from ~2 to 2·4-fold (in male and female, respectively) at 625 mg kg^{-1} b.w. and from 5·1 to 8·0-fold (in male and female, respectively) at 1250 mg kg^{-1} b.w. dose. 2β-Hydroxylase activity was affected in kidney mainly at higher dose (up to 2·2-fold, female). A CYP-dependent suppression was also recorded for 16α-hydroxylase activity (up to 81% loss in male, higher dose) and 4-androsten-3,17-dione-linked activity (~50% loss, averaged between male and female, higher dose).

Table 2. Expression of testosterone hydroxylase in kidney microsomes of mice receiving a single dose of vinclozolin.

	Vehicle only (corn oil)		Vinclozolin (625 mg $^{-1}$kg b.w.)		Vinclozolin (1250 mg $^{-1}$kg b.w.)	
Metabolites	M	F	M	F	M	F
6α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	21·97± 2·09	37·13± 3·31	39·68± 3·24**	40·44± 4·55	37·93± 3·61**	39·49± 4·54
7α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	5·07± 0·48	3·18± 0·24	3·05± 0·25**	3·07± 0·23	4·71± 0·46	2·61± 0·22*
6β-Hydroxytestosterone (pmol mg^{-1} min^{-1})	31·84± 2·94	17·09± 1·64	39·65± 3·05**	45·92± 1·38**	78·01± 7·84**	78·65± 4·91**
16α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	21·31± 1·95	12·78± 1·49	5·37± 0·47**	8·90± 0·84**	3·96± 0·41**	7·81± 0·68**
16β-Hydroxytestosterone (pmol mg^{-1} min^{-1})	75·11± 6·85	44·18± 4·32	173·12± 16·59**	148·41± 14·51**	174·62± 15·46**	147·38± 14·25**
2α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	4·75± 0·42	2·65± 0·23	8·72± 0·74**	6·33± 0·15**	24·44± 1·98**	21·40± 1·13**
2β-Hydroxytestosterone (pmol mg^{-1} min^{-1})	3·02± 0·33	2·17± 0·23	2·44± 0·21	2·83± 0·25*	5·13± 0·56**	4·84± 0·17**
4-Androsten-3,17-dione (nmol mg^{-1} min^{-1})	1·19± 0·11	0·91± 0·09	0·73 0·06	0·58± 0·04	0·64± 0·04	0·46± 0·04

Each value represents the mean ± SD of six independent experiments.

See Materials and Methods section for details and experimental procedures.

(*$p<0·05$, **$p<0·01$) Significant differences between treated groups and their respective controls, using the Wilcoxon's rank method.

The most pronounced effect of vinclozolin in the lung microsomes was a 2·2-fold increase in 2β-hydroxylase at 625 mg kg^{-1} b.w. in male (table 3). At the higher dosage used, the fungicide was more effective in inducing various hydroxylations such as 6β- (up to 2·2-fold), 16α- (up to 5·1-fold) and 16β- (up to 2·7-fold) hydroxylase activities in female CD1 mice. On the contrary, 4-androsten-3,17-dione-associated activity was significantly reduced (up to 50 % loss in male, lower dose). As vinclozolin was able to significantly induce murine CYP content in both sexes, as we recently reported (Hrelia *et al.* 1996), the increase of the various hydroxylation may be due to an inductive effect.

Expression of testosterone hydroxylases in murine liver, kidney and lung after repeated administrations of vinclozolin

The expression of testosterone oxidation in liver microsomal fraction is reported in table 4. The daily vinclozolin administration (750 mg kg^{-1} b.w.) for three consecutive days was less affective than the single treatment. The CYP 3A-associated hydroxylation was enhanced up to 2·0-fold in male animals, whereas a 2·3- and 2·9-fold increase (in male and female, respectively) was observed for 2α-hydroxylation. The most induced monooxygenase activity in the liver was the

Table 3. Expression of testosterone hydroxylase in lung microsomes of mice receiving a single dose of vinclozolin.

	Vehicle only (corn oil)		Vinclozolin (625 mg kg^{-1} b.w.)		Vinclozolin (1250 mg kg^{-1} b.w.)	
Metabolites	M	F	M	F	M	F
6α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	36·31± 3·24	10·27± 1·33	34·44± 3·72	13·68± 1·36**	47·55± 4·94**	17·80± 1·48**
7α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	3·47± 0·32	2·04± 0·18	3·31± 0·35	2·23± 0·16	5·14± 0·44*	1·81± 0·18
6β-Hydroxytestosterone (pmol mg^{-1} min^{-1})	33·75± 2·15	28·51± 2·39	34·83± 2·07	40·70± 3·58**	39·06± 3·47**	61·38± 5·29**
16α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	11·76± 1·15	14·18± 1·27	18·84± 0·76**	17·75± 0·59**	28·25± 0·31**	72·28± 0·09**
16β-Hydroxytestosterone (pmol mg^{-1} min^{-1})	72·19± 6·04	66·52± 6·48	55·20± 5·76**	62·92± 6·05	50·58± 4·82**	181·38± 7·71**
2α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	3·06± 0·28	5·65± 0·51	2·74± 0·21	7·29± 0·76**	5·73± 0·44**	7·39± 0·72**
2β-Hydroxytestosterone (pmol mg^{-1} min^{-1})	2·72± 0·23	1·57± 0·14	6·05± 0·55**	1·46± 0·12	6·47± 0·64**	1·38± 0·09
4-Androsten-3,17-dione (nmol mg^{-1} min^{-1})	1·58± 0·12	0·81± 0·07	0·75± 0·07	0·64± 0·05	0·90± 0·08	0·77± 0·07

Each value represents the mean ± SD of six independent experiments.
See Materials and Methods section for details and experimental procedures.
(*$p<0·05$, **$p<0·01$) Significant differences between treated groups and their respective controls, using the Wilcoxon's rank method.

Table 4. Expression of testosterone hydroxylase in liver microsomes of mice receiving repeated doses of vinclozolin.

	Vehicle only (corn oil)		Vinclozolin (750 mg^{-1} kg b.w. daily, for 3 days)	
Metabolites	M	F	M	F
6α-Hydroxytestosterone ($pmol\ mg^{-1}\ min^{-1}$)	80·28± 6·64	80·83± 7·91	159·32± 6·08**	114·59± 10·06**
7α-Hydroxytestosterone ($pmol\ mg^{-1}\ min^{-1}$)	15·28± 1·45	10·39± 1·15	66·25± 0·50**	78·51± 6·83**
6β-Hydroxytestosterone ($nmol\ mg^{-1}\ min^{-1}$)	0·61± 0·07	0·91± 0·07	1·24± 0·02**	1·70± 0·19**
16α-Hydroxytestosterone ($pmol\ mg^{-1}\ min^{-1}$)	38·43± 3·06	12·03± 0·98	17·87± 1·54**	13·38± 1·58
16β-Hydroxytestosterone ($pmol\ mg^{-1}\ min^{-1}$)	91·13± 8·77	103·28± 9·73	77·26± 6·39*	81·46± 8·31**
2α-Hydroxytestosterone ($pmol\ mg^{-1}\ min^{-1}$)	4·92± 0·53	4·19± 0·51	11·42± 0·92**	12·09± 0·17**
2β-Hydroxytestosterone ($pmol\ mg^{-1}\ min^{-1}$)	77·36± 5·84	85·62± 7·28	129·39± 6·39**	109·18± 8·31**
4-Androsten-3,17-dione ($nmol\ mg^{-1}\ min^{-1}$)	1·42± 0·12	1·56± 0·12	1·50± 0·14	1·21± 0·13

Each value represents the mean ± SD of six independent experiments.
See Materials and Methods section for details and experimental procedures.
(*$p<0·05$, **$p<0·01$) Significant differences between treated groups and their respective controls, using the Wilcoxon's rank method.

7α-hydroxylation (from 4·3 to 7·6-fold, in male and female, respectively). Conversely, testosterone 16α-hydroxylase activity was decreased (50 % loss) in male mice.

In the kidney, vinclozolin was able to induce several testosterone hydroxylations in a sex-dependent fashion. Indeed, only female CD1 mice were responsive to induction when probed by the selected multibiomarker of effect. Table 5 shows that 7α- (catalysed by CYP 2A1 and CYP 2B1), 6β-, 2α- and 4-androsten-3,17-dione-dependent hydroxylase activities all were significantly increased (~2·2-fold) by treatment with this pesticide. 16β-Hydroxylase activity was the most induced oxidation in kidney (up to 3·2-fold female).

In the lung, similar behaviour was observed; the female mice being the most responsive to vinclozolin modulation (table 6). Testosterone 6α- (2·5-fold), 7α- (3·7-fold), 6β- (2·8-fold), 16α- (which reflect CYP 2B9; 8·9-fold), 16β- (3·4-fold) and 2α-hydroxylase activities all were significantly ($p<0·01$) enhanced by repeated treatment.

Expression of glutathione S-transferases in murine liver, kidney and lung after repeated administrations of vinclozolin

Glutatione S-transferase activity was selected as index of phase II reactions and measured with 1-chloro-2,4-dinitrobenzene as substrate (table 7). The microsomal activity was markedly induced by this fungicide in the liver fro[illegible]

Table 5. Expression of testosterone hydroxylase in kidney microsomes of mice receiving repeated doses of vinclozolin.

	Vehicle only (corn oil)		Vinclozolin (750 mg^{-1} kg b.w. daily, for 3 days)	
Metabolites	M	F	M	F
6α-Hydroxytestosterone (nmol mg^{-1} min^{-1})	24·55± 3·08	37·24± 3·55	34·52± 3·28**	45·49± 3·89*
7α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	6·06± 0·54	4·19± 0·40	7·24± 0·49	8·92± 0·79**
6β-Hydroxytestosterone (pmol mg^{-1} min^{-1})	21·93± 2·76	22·38± 2·58	23·15± 2·17	45·62± 4·76**
16α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	16·78± 1·53	7·75± 0·69	12·86± 1·33*	4·27± 0·46
16β-Hydroxytestosterone (pmol mg^{-1} min^{-1})	71·11± 6·83	41·31± 3·82	89·54± 7·61**	131·39± 12·37**
2α-Hydroxytestosterone (pmol mg^{-1} min^{-1})	4·23± 0·38	4·55± 0·47	5·37± 0·56	10·64± 1·35**
2β-Hydroxytestosterone (pmol mg^{-1} min^{-1})	2·66± 0·23	2·94± 0·33	3·09± 0·28	2·44± 0·27
4-Androsten-3,17-dione (nmol mg^{-1} min^{-1})	1·09± 0·09	1·27± 0·11	0·91± 0·08	2·85± 0·24**

Each value represents the mean ± SD of six independent experiments.
See Materials and Methods section for details and experimental procedures.
(*p<0·05, **p<0·01) Significant differences between treated groups and their respective controls, using the Wilcoxon's rank method.

male and female, respectively) and in kidney from 3·3 to 3·9-fold (in male and female, respectively). In contrast, it should be noted that the lung was not responsive to vinclozolin induction. Indeed, no significant differences between controls and treated groups were seen in either male or female CD1 mice. Because some adenoma were found in the lung only, these data could be importantly pointing out the role of the ratio between activating/detoxificating activities in determining the organospecific toxic effect of a xenobiotic.

Discussion

Chronic oral and toxicity investigations on vinclozolin reported in EPA's files seem to indicate the low toxicity of the chemical (EPA 1985, 1987). Short-term tests for mutagenicity failed, in general, to show genotoxic activity in bacterial cell culture and whole animal bioassays. However, a weak oncogenic effect on mice and reproductive and developmental toxicity in rats have been reported (EPA 1985, Von Ravenzwaay 1992, Kelce *et al.* 1994).

Here, we studied the ability of this fungicide to induce changes in selected carcinogen- metabolizing enzymes known to interfere with non-genotoxic tumorigenesis. Somatic mutation theory, dealing with chemical activity at proto-oncogenes or tumour-suppressor gene levels, cannot explain [illegible] of

Table 6. Expression of testosterone hydroxylase in lung microsomes of mice receiving repeated doses of vinclozolin.

	Vehicle only (corn oil)		Vinclozolin (750 mg^{-1} kg b.w. daily, for 3 days)	
Metabolites	M	F	M	F
6α-Hydroxytestosterone ($nmol\ mg^{-1}\ min^{-1}$)	32·81± 3·27	24·34± 2·52	43·56± 4·55**	60·22± 5·81**
7α-Hydroxytestosterone ($pmol\ mg^{-1}\ min^{-1}$)	4·11± 0·32	3·71± 0·35	7·19± 0·65**	13·85± 1·25**
6β-Hydroxytestosterone ($nmol\ mg^{-1}\ min^{-1}$)	37·97± 3·55	32·84± 2·82	29·42± 2·53**	91·48± 6·74**
16α-Hydroxytestosterone ($pmol\ mg^{-1}\ min^{-1}$)	16·24± 1·24	12·39± 1·54	24·58± 2·61**	111·04± 0·96
16β-Hydroxytestosterone ($pmol\ mg^{-1}\ min^{-1}$)	64·75± 6·15	75·84± 7·16	92·03± 8·21**	258·31± 16·26**
2α-Hydroxytestosterone ($pmol\ mg^{-1}\ min^{-1}$)	3·99± 0·36	3·19± 0·29	5·12± 0·56*	9·07± 0·87**
2β-Hydroxytestosterone ($pmol\ mg^{-1}\ min^{-1}$)	2·47± 0·19	1·83± 0·22	2·63± 0·31	1·74± 0·15
4-Androsten-3,17-dione ($nmol\ mg^{-1}\ min^{-1}$)	1·62± 0·11	1·19± 0·07	1·25± 0·09	1·66± 0·18

Each value represents the mean ± SD of six independent experiments.
See Materials and Methods section for details and experimental procedures.
(*p<0·05, **p<0·01) Significant differences between treated groups and their respective controls, using the Wilcoxon's rank method.

Table 7. Expression of glutathione s-transferase in murine liver, kidney and lung of vinclozolin treated animals.

	Untreated (vehicle) (corn oil)		Vinclozolin (750 mg^{-1} kg b.w. daily, for 3 days)	
Activity ($nmol\ mg^{-1}\ min^{-1}$)	M	F	M	F
Liver	1740± 196	1705± 179	6790± 382**	8980± 407**
Kidney	732± 48	684± 59	2441± 125**	2663± 158**
Lung	987± 83	1·015± 102	975± 96	992± 84

Each value represents the mean ± SD of six independent experiments.
See Materials and Methods section for details and experimental procedures.
Significant differences between treated groups and their respective controls, using the Wilcoxon's rank method (**p<0·01).

non-genotoxic carcinogens, which represent more than 50 % of total carcinogens (IARC 1991, Kitchin *et al.* 1993, Rabbits 1994). It is, indeed, very important to recognize that other biological activity of a xenobiotic than mutagenesis may be more important in the induction of cancer. Various mechanisms have been proposed for non-genotoxic carcinogenesis such as :

(a) unspecific toxic effect from administration of high doses of the test compound (MTD) leading to cell death and subsequent stimulation of mitosis (Hai 1991);

(b) facilitation of the biliary clearance of thyroid hormone T4 by thyroxine glucuronyl-transferase induction: this decrease of circulating T4 leads to secretion of thyroid-stimulating hormone and, consequently, thyroid hypertrophy and hyperplasy eventually resulting in thyroid tumours (Shaw and Jones 1994);

(c) pleiotropic response (i.e. simultaneous induction of phase I, phase II and other non-correlated enzymes by a single chemical), oxidative stress (associated to CYP overexpression; it can act at all multistep levels of carcinogenesis) and cocarcinogenesis (i.e. increased bioactivation of ubiquitous xenobiotics by a more active CYP-dependent system) (Hai 1991, Kitchin *et al.* 1993, Paolini *et al.* 1994, 1996);

(d) promotion (linked to oxygen radical yielding by means of CYPs enhancement) and induction regulation of cell proliferation (associated to the increase of superoxide release by CYP isozymes) (Schultze-Hermann *et al.* 1988, Shi and Yager 1989, Paolini *et al.* 1996);

(e) peroxisomal proliferation (depending upon CYP inducers) (Conway *et al.* 1990);

(f) $\alpha 2$-globulin accumulation in kidney tubule cells (Eldrige *et al.* 1990).

Thus, the points (c), (d) and (e) are all related to CYP induction.

In this investigation vinclozolin exhibited a complex pattern of CYP modulation (induction and suppression), typical of ergosterol biosynthesis inhibiting fungicides (Ronis *et al.* 1994). This pesticide, being able to induce different testosterone hydroxylations should be considered as an 'unspecific' enzyme inducer. However, CYP 3A, the most representative enzyme present in human liver, was significantly affected in all tissue considered. Whereas testosterone 16α-hydroxylase activity, associated with CYP 2B9, was induced only in the lung, mainly in female CD1 mice. The other induced hydroxylations were not associated to specific isoforms in mice. To our knowledge, this is the first report leading with induction of CYP 3A by vinclozolin in the lung. These findings point out the importance of using different substrates in probing various CYP in terms of specificity as well as sensitivity of the used methodology. Indeed, the use of the common probes to various CYPs, failed to detect in rodent lung any significant induction by vinclozolin (Hrelia *et al.* 1996). The observed different CYP expression in different tissues and sex could imply:

(a) different induction mechanisms;

(b) alterations in competing pathways involved in the disposition of either parent compounds or metabolites;

(c) differences in the substrate specificity. CYPs from various tissues may elicit different metabolism, due to the 'altered' substrate specificity of the involved isoform, together with the contribution, for that substrate, of other CYPs.

The observed sex dependent differences in enzymes induction/suppression could be explained in terms of quantitative or qualitative differences in the CYP isoenzymes which are under the control of sex hormones.

Repeated treatment with vinclozolin did not substantially modify the pattern and extent of CYP induction, at least in the liver. Indeed, kidney and lung showed a clear sex-dependent modulation, the female being the most responsive to induction by this fungicide. In the lung, together CYP 3A and CYP 2B9, CYP 2A1 and CYP 2B1 (associated to 6α-hydroxylation) were also induced by treatment.

We considered the importance of the ratio between activating and detoxificating mechanisms in expressing chemical toxicity in a given tissue, as well as the high susceptibility of lung in female mice to certain forms of malignancy (EPA 1985). In the present work we studied the behaviour of glutathione S-transferase activity as index of post-oxidative reactions. The activity was markedly increased by repeated vinclozolin treatment in both liver and kidney but it was unaffected in the lung. The phase I/phase II ratio was thus unchanged by treatment in liver and kidney, the fungicide being able to induce both oxidative and post-oxidative reaction rates. In contrast, in the lung, vinclozolin was able to selectively induce the CYP-catalysed metabolism in female mice only (organospecific monofunctional inducer). Bioactivation is a key step in chemical carcinogenesis and CYP machinery plays a key role in this respect. The perturbation of carcinogen-metabolizing enzymes induced by vinclozolin in the lung of female mice could contribute to the documented risk of cancer. It should be taken into account that the deleterious effects from vinclozolin exposure can also be mediated by the antiandrogenic M1 and M2 metabolites. An alteration of the metabolic pathway in terms of either induction or inhibition can lead, therefore, to an alteration of the antiandrogenic activity.

Whatever the inductive mechanism, so far not well defined for different isozymes (Paolini *et al.* 1995), CYP modulation can determine serious toxicological and mutagenic/carcinogenic consequences. Altered enzyme expression may be related to variation in kinetic factors that affect tissue dosimetry, leading to cotoxicity and comutagenicity/carcinogenicity (IARC 1991). The inductive or inhibitory effects, in addition to the alteration of endogenous metabolism where these catalysts are physiologically associated, can exert a booster phenomenon towards the bioactivation of ubiquitous environmental pollutants. It should be point out that CYP inducers may also act as promoting agents in chemical carcinogenesis by either a pleiotropic response (Lubet *et al.* 1992) or oxygen radical overproduction (Cerutti 1985, Paolini *et al.* 1996b). This increased generation of oxygen centred radicals, which is strictly associated to CYP induction, can impose a 'prooxidant status' which point toward cancer (Bast 1986). Furthermore, cell transforming activity was reported by Perocco *et al.* (1993) in the BALB/c 3T3 test. Since the cell transformation assay can detect both carcinogens that act by a primary genotoxic mechanism and those that act by an alternative non-genotoxic mechanism (IARC 1991), transforming activity may indicate that vinclozolin might promote to a more malignant phenotype a background of cells initiated by the fungicide itself, or pre-existing in the BALB/c 3T3 population. A significant increase in the rate of preneoplastic glutathione S-transferase placental form positive foci in the rat liver was also found by Ito *et al.* (1994). Concerning the inhibition of metabolizing enzymes, for their ability to simultaneously reduce the activation of some chemicals but also reduce the detoxyfication of countless other xenobiotics, is a detrimental mechanism. Whilst inhibition can decrease the bioactivation of some pretoxics and premutagens/precarcinogens, at the same time it decreases also the detoxification of other innumerable foreign compounds (Paolini *et al.* 1998).

In conclusion, the data presented in this investigation can contribute to the definition of the possible toxicological consequences for human population potentially exposed to vinclozolin. This is particularly important after long-term co-exposure to xenobiotics specifically metabolized by the induced CYP isoforms (CYP 3A, 2B1, 2B9 and 2A1) in the lung. However, considering the high doses

(over 625 mg kg^{-1} b.w.) at which this pesticide caused toxicological effect in mice, compared with the dose permitted in humans (ADI 0·025 mg kg^{-1} day^{-1}; EPA 1985), the cocarcinogenic risk seems to be limited.

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