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Evaluation of the hepatotoxicological effects of a drug in an in vivo/in vitro model

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Abstract. Both in vivo and in vitro models have certain disadvantages for the study of the chronic hepatotoxicity of drugs. The aim of this work was to evaluate a new approach based on an in vivo/in vitro model. After chronic in vivo treatment of rats with Vincamine and Vindeburnol (an eburnamenine derivative which exhibits hepatotoxic properties in man) liver cells were isolated, and functional and metabolic disorders (metabolic utilization of fructose and protein biosynthesis) were studied to determine injury. The results showed no modification of blood parameters, but a direct relationship between the dose of Vindeburnol administered in vivo and the metabolic disorders observed in vitro, evidencing the high sensitivity and reliability of this model.
Key words. Hepatotoxicity; in vivo/in vitro model; isolated hepatocytes; α1-AGP and albumin mRNAs.

The hepatotoxic effects of drugs are usually studied in animals by the assay of blood markers of liver damage such as aminotransferases, alkaline phosphatases and bilirubin. However, the lack of sensitivity of these tests can lead to an underestimation of toxicological effects. Therefore in vitro studies with isolated hepatocytes have been used increasingly over the last ten years [1–3], although they also have major shortcomings. Indeed, freshly isolated hepatocytes may respond atypically to chemicals added in vitro, due to morphological and biochemical trauma inflicted during isolation [4]. In addition, conventionally cultured hepatocytes normally undergo rapid phenotypic alterations, including large decreases in several drug-metabolizing enzymes [5]. Finally, drug administration in vitro does not necessarily correspond to conditions encountered in vivo [6].

Recently, Mac Donald et al. [7] developed an in vivo/in vitro model to study the cytoprotective action of cystamine in liver necrosis caused by galactosamine. We have developed a similar in vivo/in vitro model for evaluating the hepatotoxic potential of various drugs. Vindeburnol and Vincamine were administered to rats for six days, and liver insult was then evaluated in hepatocytes isolated from these animals by determining alterations of functional and metabolic capacities, i.e. protein synthesis and carbohydrate utilization.

This experimental procedure is relatively simple to perform, is sensitive enough to reveal liver damage in the absence of changes in blood markers, and permits a direct relationship to be established between drug doses administered in vivo and their effects measured in vitro using isolated hepatocytes.

Materials and methods

Fifty male Sprague-Dawley rats (220–250 g) were fed ad libitum with laboratory chow. The animals were divided into five groups, three of which received 30, 60 and 120 mg/kg/day of Vindeburnol per os for six days. The fourth group received 120 mg/kg/day of Vincamine by the same route, while controls received only the vehicle (1% carboxymethyl cellulose).

Chemicals: Vindeburnol (RU 24722) and Vincamine were gifts from the Centre de Recherches Roussel Uclaf (Romainville, France).

Blood parameters: Immediately before sacrifice, blood was collected from the abdominal aorta for the determination of biological markers of hepatic injury, i.e. aminotransferases, γ-glutamyl transpeptidase (γ-GT), alkaline phosphatase (all with fully enzymatic methods according to the recommendations of the Société Française de Biologie Clinique), and total bilirubin. Albumin and α_1-acid glycoprotein (α_1-AGP), two hepatocyte-synthe-

sized plasma proteins, were determined using the radial immunodiffusion method of Mancini[8].

Isolated hepatocytes were prepared according to the technique of Berry and Friend[9], as modified by Davy et al.[10]. In order to increase viability, the hepatocyte suspension (12 ml containing about 1.5×10^6 cells/ml) was incubated in collagen-coated plates at +37 °C in a humidified atmosphere of 5% CO_2/95% air, with Krebs Ringer bicarbonate buffer (KRBB), pH 7.4[11]. After 30 min the medium was discarded, cell viability was checked, and KRBB or Eagle's minimum essential medium (EMEM) added. After further incubation for 3 or 4 h, adherent cells were resuspended in collagenase buffer, and viability and cell numbers were assessed using the trypan blue exclusion test.

For the evaluation of glucose and lactate production, aliquots of liver cells were incubated in KRBB for three hours with or without 10 mM fructose. The concentrations of fructose, glucose and lactate in the supernatant were measured one, two and three hours later. Glucose was determined using the glucose oxidase method, and lactate using the lactic dehydrogenase method. Fructose was measured using Selivanof's reaction. Results were first calculated as µmol of glucose or lactate released and µmol of fructose consumed per 10^6 cells, then as % inhibition relative to controls as a function of the Vindeburnol dose. For the measurement of albumin and α1-AGP secretion, aliquots of liver cells were incubated for 4 h in EMEM according to the method of Davy et al.[10]. The proteins were measured in the supernatant by means of sandwich-type immunoenzymatic methods[12].

Liver specimens (500 mg) from 6 rats (2 controls, 2 treated with the highest dose of Vindeburnol and 2 treated with 1 ml of turpentine to induce a maximal inflammatory response) were collected and immediately frozen in sterile tubes placed for a few minutes in a dry-ice ethanol bath, and then stored at −80 °C until use.

Isolation of RNA and Northern blot analysis: Total cellular RNA was extracted from frozen pieces of rat liver by the guanidine isothiocyanate/cesium chloride method[13] using a Beckman SW 55 rotor for 16 h at 30000 rpm. Heat-denaturated RNA samples were run for 16 h at 2 volts/cm as previously described[14], in 1.2% agarose gels containing 2.2 M formaldehyde. RNA was blotted onto Biodyne A nylon membranes (Pall Ultrafine Filtration Corporation) by Northern capillary transfer for at least 48 h. After transfer, the filters were air-dried and baked at 80 °C for 2 h, then prehybridized for at least 3 h at 42 °C in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.1% SDS, 50 mM sodium phosphate, pH 6.5, and 250 µg/ml denatured herring sperm DNA. Recombinant plasmids containing insert encoding rat α1-AGP or albumin mRNA were used to probe the Northern blots. The RR1 strain of *Escherichia coli* containing the α1-AGP recombinant plasmid was obtained from Taylor[15], while the two strains of *E. coli* containing the 3′ and 5′ ends of the rat albumin gene were cloned in Sala-Trepat's laboratory[16]. Plasmid DNA containing the α1-AGP and albumin inserts were labelled by nick-translation using (α-^{32}P) dCTP. The specific activity of the plasmid DNAs was about 10^8 cpm/µg. Hybridization was carried out for 48 h at 42 °C under the same conditions as for prehybridization. Filters were washed under high stringency using the Milliblot-Blot processor system (Millipore) at room temperature with 1 l of 2X SSC and 0.1% SDS, and then at 50 °C with 1 l of 0.1X SSC and 0.1% SDS. The wet filters, enclosed in Saran film, were exposed to hyperfilm MP (Amersham) at −80 °C using Lightning Plus intensifying screens (Dupont Cronex).

Dot blot analysis: Messenger RNAs for α1-AGP and albumin were quantified in total cellular and poly(A)$^+$ RNA selected through two cycles of oligo(dT)-cellulose chromatography. Using serial half-dilutions, 10 µg–625 ng total cellular RNA, and 5 µg–313 ng poly(A)$^+$ RNA, were spotted onto nylon membranes which were then processed as for Northern blots. Autoradiographs were scanned by means of photometric densitometry (Sebia) and the results expressed as relative densities of total cellular and poly(A)$^+$ RNA.

Results

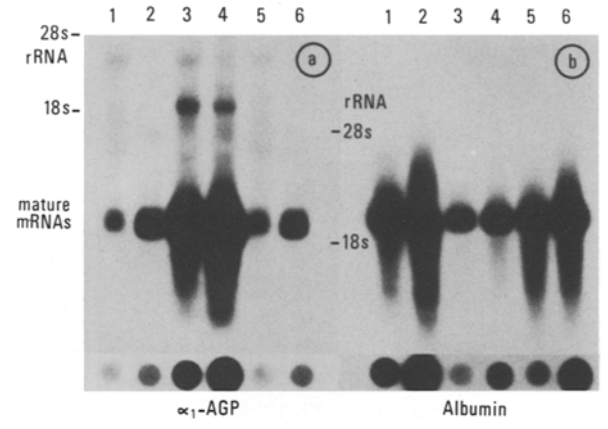
1) *In vivo studies.* Table 1 shows blood aminotransferases, alkaline phosphatase and γ-GT activities, together with bilirubin, α_1-AGP and albumin levels in the five groups of animals. As expected, there were no significant differences between treated and control groups.

Hepatic α1-AGP and albumin mRNAs. Figure 1 shows Northern and dot blot analysis of hepatic mRNA for

Table 1. Blood parameters in control rats and rats treated with Vincamine or Vindeburnol for six days

	Control n = 10	Vincamine 120 mg/kg n = 10	Vindeburnol 120 mg/kg n = 10	60 mg/kg n = 10	30 mg/kg n = 10	
ASAT[a] (IU/l)	58 ± 24	54 ± 20	51 ± 21	48 ± 14	43 ± 12	NS
ALAT[b] (IU/l)	30 ± 7	24 ± 7	26 ± 11	26 ± 6	23 ± 4	NS
PAL[c] (IU/l)	228 ± 29	237 ± 50	226 ± 59	228 ± 54	215 ± 28	NS
γGT[d] (IU/l)	2 ± 1	2 ± 2	2 ± 2	2 ± 1	2 ± 1	NS
T-Bili[e] (µmol/l)	17 ± 1	19 ± 4	21 ± 6	18 ± 2	17 ± 1	NS
Albumin (g/l)	40.6 ± 4.8	39.7 ± 3.7	39.9 ± 3.8	40.2 ± 4.1	42.2 ± 2.5	NS
α_1-AGP[f] (mg/l)	119 ± 48	127 ± 46	111 ± 60	145 ± 56	118 ± 31	NS

Mean ± SE; NS: Not significant. [a] Aspartate aminotransferase; [b] alanine aminotransferase; [c] alkaline phosphatase; [d] gamma glutamyl transpeptidase; [e] total bilirubin; [f] α_1-acid glycoprotein.



dots	1	2	3	4	5	6	1	2	3	4	5	6
total RNA	460		5220		500		3450		525		1580	
poly(A)+ mRNA		2080		20760		2000		14220		3560		9990

Figure 1. Northern and dot blot analysis of hepatic α1-AGP and albumin mRNAs. Slots and dots 1, 3 and 5 correspond to total cellular mRNA from controls, turpentine- and Vindeburnol-treated rats, respectively. Slots and dots 2, 4 and 6 correspond to the poly(A)$^+$ mRNA enriched from the same RNA samples by two cycles of oligo (dT)-cellulose chromatography. Each RNA sample analyzed was from two pooled RNA samples extracted from two different rat livers. Integration values of dots are given for 1 µg of total and poly(A)$^+$ RNA. Values were calculated from the mean integration of dots giving proportional densities.

α1-AGP (part a) and albumin (part b) from control (slots 1, 2), turpentine-treated (slots 3, 4) and Vindeburnol-treated rats (slots 5, 6). Control and Vindeburnol-treated rats showed no significant difference in the total and poly(A)$^+$ hepatic α1-AGP mRNA (fig. 1a, slots 1, 2 and 5, 6). In contrast, the turpentine-treated rats showed an 11-fold increase in both total and poly(A)$^+$ α1-AGP mRNA (fig. 1a, slots 3 and 4). Vindeburnol-treated rats showed a 42% decrease in total and poly(A)$^+$ hepatic albumin mRNA (slots 5, 6), relative to control rats (slots 1, 2), while the inflamed rats showed an 80% decrease (fig. 1b, slots 3, 4).

2) *In vitro studies.* The proportions of viable and adherent cells were comparable in the five groups at the beginning and end of the incubation period, showing that the in vivo treatment and isolation procedure had no major effects on these parameters (table 2). Viability was similar (80–84%) with the different media (KRBB, KRBB + fructose or EMEM).

Metabolic utilization of fructose. There were no statistical differences between control and Vincamine-treated rats throughout the incubation period (0–3 h), in terms of fructose consumption, or lactate and glucose release into the medium (not shown). Vindeburnol treatment induced a significant, dose-related, inhibition of these parameters compared to control rats (fig. 2). As all the parameters varied linearly throughout the incubation period, the results shown in figure 2 are those for an incubation time of 3 h, at which the effect was most marked. Only a slight decrease (15%) in glucose release occurred following treatment with the lowest dose of Vindeburnol (30 mg/kg, fig. 2c, $p < 0.05$). With the intermediate dose (60 mg/kg) the effects were more marked, with a 10% decrease in fructose consumption (fig. 2a, $p < 0.05$) and a 16% inhibition of glucose release (fig. 2c, $p < 0.05$). The highest dose of Vindeburnol (120 mg/kg) induced a strong decrease in the three parameters measured: fructose consumption was reduced by 19% (fig. 2a, $p < 0.05$), lactate release by 15% (fig. 2b, $p < 0.05$) and glucose release by 31% (fig. 2c, $p < 0.01$).

Albumin and α_1-AGP secretion. No significant effect of Vincamine or Vindeburnol (30 mg/kg) on albumin secretion was observed after 4 h incubation in EMEM (fig. 3a). There was a slight decrease (−15%, $p < 0.05$) with the intermediate dose of Vindeburnol, and a marked reduction with the highest dose (−33%, $p < 0.01$). Individual variability in α1-AGP secretion between the ani-

Table 2. Adherence and viability before and after incubation of hepatocytes isolated from control rats and rats treated with Vincamine or Vindeburnol for six days

	Control	Vincamine 120 mg/kg	Vindeburnol 120 mg/kg	60 mg/kg	30 mg/kg	
Before incubation						
Viability after isolation (%)	78 ± 5	82 ± 3	79 ± 3	84 ± 4	81 ± 6	NS
Hepatocytes added to dishes (×10^6)	19.2 ± 1.2	18.7 ± 0.9	17.4 ± 1.6	18.1 ± 1.2	17.9 ± 1.4	NS
After 4 h incubation						
KRBB[a] and KRBB + fructose	n = 20	n = 20	n = 20	n = 20	n = 20	
Cells (×10^6)	10.7 ± 3.4	10.9 ± 3.4	9.2 ± 2.4	9.6 ± 1.8	9.1 ± 1.6	NS
Adherence (%)	56 ± 8	58 ± 7	53 ± 6	53 ± 5	51 ± 4	NS
Viability (%)	84 ± 4	84 ± 4	84 ± 4	84 ± 5	83 ± 6	NS
EMEM[b]	n = 15	n = 15	n = 15	n = 15	n = 15	
Cells (×10^6)	9.3 ± 2.0	9.5 ± 2.5	8.9 ± 1.7	9.3 ± 2.5	9.8 ± 2.7	NS
Adherence (%)	48 ± 5	51 ± 6	51 ± 4	51 ± 6	54 ± 6	NS
Viability (%)	80 ± 5	80 ± 4	82 ± 5	82 ± 5	82 ± 3.9	NS

Mean ± SE; NS: Not significant. [a] Krebs Ringer bicarbonate buffer; [b] Eagle's minimum essential medium.

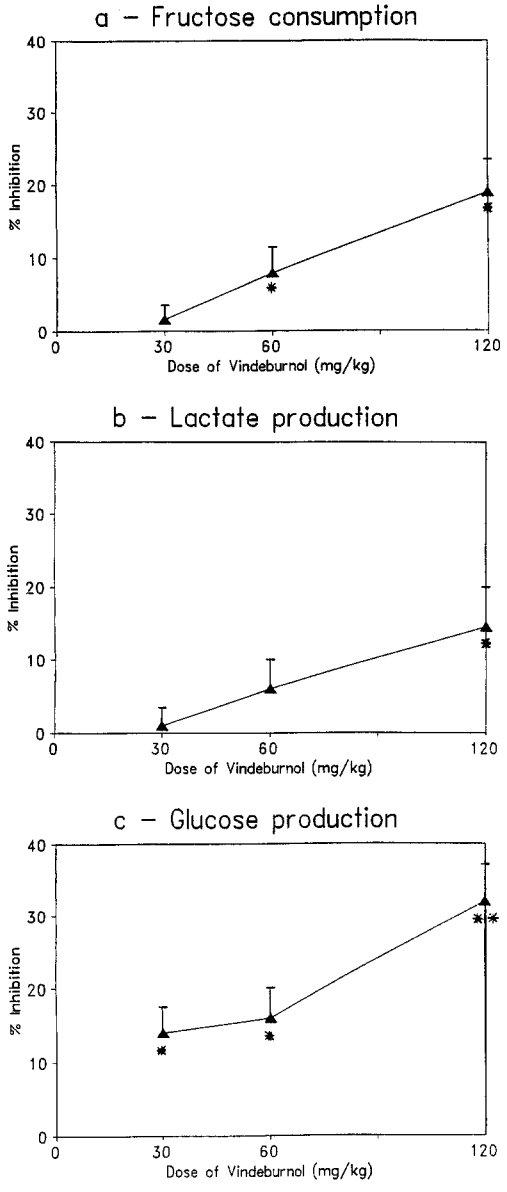


Figure 2. Inhibition %, as compared to control rats, of fructose consumption (*a*), and lactate (*b*) and glucose (*c*) production by hepatocytes isolated from rats treated with 30, 60 and 120 mg/kg Vindeburnol, after 3 h of incubation in KRBB + fructose (10 mM).
(* $p < 0.05$; ** $p < 0.01$)

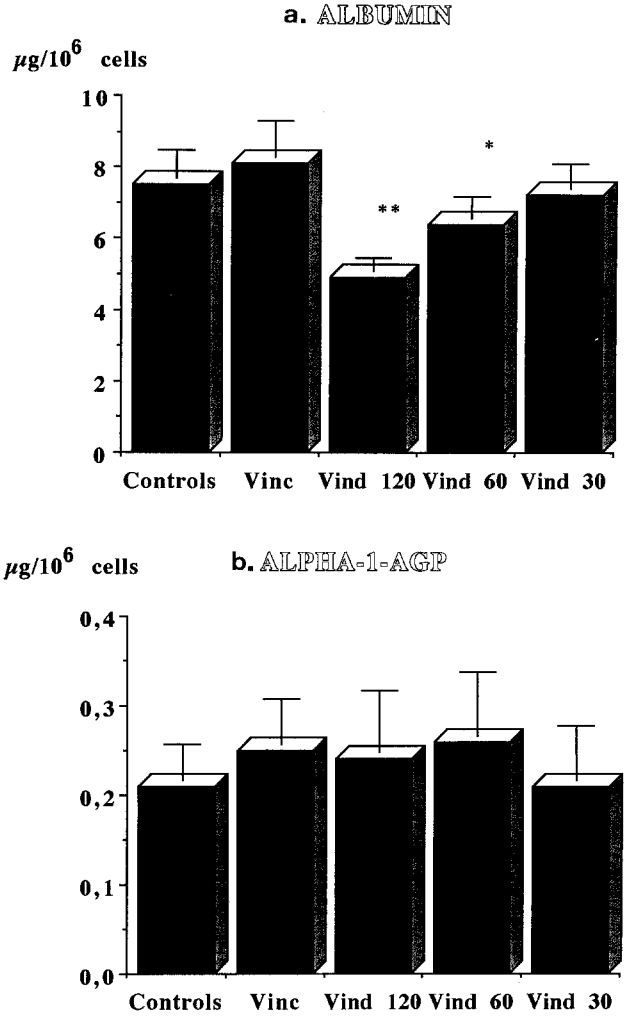


Figure 3. Albumin and α_1-AGP secretion by hepatocytes isolated from rats treated with Vincamine (120 mg/kg) and Vindeburnol (30, 60 and 120 mg/kg) after 4 h of incubation.
(* $p < 0.05$; ** $p < 0.01$)

mals was much higher than for albumin, so the changes observed were not statistically significant (fig. 3 b). Interestingly, the levels of both albumin and α1-AGP secretion by hepatocytes from rats treated with the highest dose of Vindeburnol correlated perfectly with corresponding hepatic mRNA levels, as shown by Northern and dot blotting (fig. 1).

Discussion

In vivo/in vitro models have previously been used in metabolic studies and to investigate the mechanism of the protective effects of drugs like cystamine towards hepatocytes [7, 17]. Here, we investigated whether such an approach could be used to screen drugs for hepatotoxicity. Vindeburnol [18] has occasionally been reported to induce increases in aminotransferases in man, while Vincamine is not thought to be toxic for the liver, although marked alterations of the tight junctional system have been observed [19]. The doses of Vindeburnol used in this study (30, 60, 120 mg/kg) were relatively high, with an LD_{50} per os in rats of 177 mg/kg and therapeutic doses in man of 80 mg/day, but this allowed the treatment to be administered over a short period. Preliminary studies were performed to avoid doses which induce patent liver damage, revealed by increases in blood markers such as aminotransferases. In order to simplify the model, we measured alterations of metabolic capacities of isolated liver parenchymal cells using only two parameters which are highly specific and sensitive. The ability to metabolize fructose was evaluated by studying its consumption, together with the concomitant release of glucose and lactate, as extensively described in the literature [20]. The syn-

thesis of specific proteins such as albumin and α_1-acid glycoprotein is also well documented in normal and turpentine-treated rats[10]. These proteins were chosen because they are considered as major biological markers of liver disease. Severe hepatocellular insufficiency is very often associated with a decrease in serum albumin levels, and all inflammatory reactions are accompanied by an enhancement of the acute-phase protein levels, particularly α_1-AGP. The immunoenzymatic method used to measure these proteins was highly sensitive and the results correlated well with those obtained using labeled amino acids (results not shown).

In the present study, Vindeburnol induced an inhibition of albumin secretion, with decreases in fructose consumption and glucose and lactate production, particularly at 120 mg/kg. Following Vincamine treatment, values were similar to those reported with hepatocytes isolated from untreated rats[10,21]. In the absence of modifications of blood parameters in Vindeburnol-treated rats, the inhibition of specific hepatocyte functions in vitro clearly demonstrates the high sensitivity of the model for the parameters selected. The lack of effect of Vindeburnol on serum albumin levels is not surprising, given the potent adaptative mechanisms involved in maintaining constant levels of this protein in the blood. Nevertheless, the effect of Vindeburnol on albumin synthesis was clearly confirmed by the decreased levels of hepatic albumin mRNA. The absence of significant modifications of α_1-AGP secretion by isolated hepatocytes was also confirmed by the lack of effect of Vindeburnol on α_1-AGP mRNA levels in the liver.

One problem concerning the use of freshly isolated cells in hepatotoxicological studies is that the isolation procedure can cause morphological and biochemical trauma, which can require a recovery period before experimentation. However, in the in vivo/in vitro model, it is essential to test the hepatocytes as soon as possible after isolation, in order to detect functional disorders induced by the drugs, and 'memorized' temporarily by the cells. The drug-induced modifications of specific functions observed here at the isolated hepatocyte level cannot be attributed to a direct cytotoxic effect of Vindeburnol, because attachment and viability, measured at the beginning and end of the incubation period, were similar to control values, regardless of the medium. These data do not suggest that the isolation procedure used is selective against cells partially damaged by the drug in vivo.

Taken together, our results suggest a hepatotoxic effect of Vindeburnol, although the mechanism remains to be determined. The relationship between the dose administered in vivo and the intensity of the effects observed in vitro strongly suggests that Vindeburnol, or a reactive metabolite, could directly alter liver cell constituents (by covalent binding for example) and that an immunoallergic mechanism can be ruled out.

In conclusion, this in vivo/in vitro approach can be used successfully to test for possible hepatotoxic effects, although further studies with other drugs are required to validate its screening potential.

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