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THE EFFECT OF CIMETIDINE AND RANITIDINE ON PARACETAMOL GLUCURONIDATION AND SULPHATION IN CULTURED RAT HEPATOCYTES

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Abstract—Cimetidine and ranitidine have been investigated for their ability to inhibit conjugation reactions in cultures of rat hepatocytes. Neither compound had any appreciable effect on rates of paracetamol sulphation. However, both cimetidine and ranitidine inhibited the glucuronidation of paracetamol in a dose-dependent manner. No adverse effects on cellular viability were noted utilizing enzyme leakage (lactic dehydrogenase) or protein synthesis measurements. The kinetics of inhibition by ranitidine were studied in more detail. At 0.25 mM ranitidine, the inhibition appeared to be purely competitive.

However, at higher concentrations decreases in V_{max}^{app} were noted suggesting a more complex mechanism of inhibition. The relevance to inhibition *in vivo* by cimetidine and ranitidine and possible interactions between paracetamol and these histamine H_2-receptor antagonists are discussed.

The interactions of cimetidine [1] and ranitidine [2] with cytochrome P450 have been well documented and have been attributed to the formation of type II ligand binding spectra with cytochrome P450 haemoprotein. A number of studies [1–3] have indicated that in comparison with cimetidine, ranitidine is far less potent at interacting with the cytochrome P450 system.

However, many other enzyme systems are involved in the biotransformation of drugs in the body but much less data are available concerning the potential for interaction of cimetidine or ranitidine with these other routes of metabolism.

Glucuronidation and sulphation are major pathways in the biotransformation and elimination of a wide variety of lipid-soluble drugs, chemicals and endogenous compounds. Accumulating evidence suggests that the activity of these pathways may be under complex control including, in the case of glucuronidation, the amount and composition of multiple enzyme forms of glucuronyl transferase, the latency of the firmly membrane-bound enzyme, activators and inhibitors and the concentration of substrates at the active site of the enzyme. Moreover, both of these pathways have been demonstrated to be highly sensitive to cofactor levels within the cell [4]. We decided therefore, that in order to study the effects of ranitidine and cimetidine on these major conjugation pathways, cultured rat hepatocytes provide an appropriate model system for our investigations.

The aim of this investigation is to study the effects of cimetidine and ranitidine on both sulphation and glucuronidation reactions as measured by paracetamol conjugation utilizing cultured rat hepatocytes.

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MATERIALS AND METHODS

Cimetidine hydrochloride and ranitidine hydrochloride were used as lyophilized powders from commercial preparations. Collagenase (type IV), Deoxyribonuclease (type I), Insulin (bovine pancreas), bovine serum albumin (BSA, fraction V) and trypan blue were obtained from Sigma (Poole, Dorset, U.K.). Williams Medium E (WME) was obtained from Flow (Irvine, U.K.). Newborn calf serum, L-glutamine, penicillin, streptomycin and neomycin from GIBCO (Uxbridge, U.K.). Soluble collagen was obtained from Worthington (Millipore, Corp., Paisley, U.K.). Multiwell plates (35 mm) were purchased from Sterilin (Teddington, Middlesex, U.K.). Radioactive paracetamol (*p*-hydroxy[ring-3,5-^{14}C] acetanilide) and L-[U-^{14}C]-leucine were obtained from the Radiochemical Centre (Amersham, Bucks., U.K.). Rats (Wistar, male) were obtained in the range 180–200 g from the SK&F colony and were housed on grade 6 greenwood granules in polypropylene cages. Free access to tap water and PRD pellets (Labsure, Poole, Dorset, U.K.) was provided.

Hepatocytes were prepared by the technique of Strom *et al.* [5]. Rats were killed by cervical dislocation, the livers removed and stored under ice-cold saline. Lobes were removed with a scalpel and a major vessel of the lobe cannutated with a polyethylene catheter (16 gauge, Medicut). Perfusion was started immediately at a flow rate of 6 ml/min and resulted in blanching of the majority of the lobe. The perfusate consisted of NaCl (142 mM), KCl (7 mM), HEPES (10 mM, pH 7.4) and EGTA (40 μM). After 3–5 min, the perfusate was changed to NaCl (142 mM), KCl (7 mM), HEPES (10 mM, pH 7.4), $CaCl_2$ (1 mM) and collagenase (1 mg/ml) and maintained on a recycle mode for 5–7 min. A Watson–Marlow 502 pump with a multichannel head was used to maintain flow and enabled several lobes

to be perfused simultaneously. Temperature at the lobe was maintained at 37° by means of a standard water bath set at 40°, a 3° temperature loss occurring along the perfusion tubing.

The lobe was then removed from the perfusion apparatus and placed in a cold buffer [NaCl (142 mM), KCl (7 mM), HEPES (10 mM, pH 7.4) containing BSA (1% w/v)]. The capsule was then gently folded back and the hepatocytes released by gentle agitation of the lobe. The cell suspension was then filtered through nylon mesh (64 μM) and centrifuged to remove debris and reticulo-endothelial cells. Centrifugation was repeated 3 times. Hepatocytes were counted in a haemocytometer in the presence of 0.04% trypan blue. Yields of 30–40 × 10^6 cells were commonly achieved from each lobe with a viability in excess of 85%.

Hepatocytes were quickly diluted into culture medium consisting of WME containing L-glutamine (4 mM), penicillin (100 IU/ml), neomycin (100 μg/ml), insulin (0.02 IU/ml) and newborn calf serum (10% v/v). The cell suspension was then seeded on to 35 mm wells (Sterilin) which had been coated with soluble collagen. The culture dishes were introduced into a 37° incubator (T. R. Heraeus) containing 5% CO_2 in a water saturated atmosphere. Cell attachment could be detected after about 1 hr in culture. Cells were maintained in culture for two days before the initiation of experimental protocols.

Paracetamol metabolism was measured in the cultured rat hepatocyte preparation by directly dissolving the substrate in WME containing L-glutamine (4 mM), penicillin (100 units/ml), Streptomycin (100 μg equivalent/ml) and neomycin (100 μg/ml). The incubation was performed at 37° in a humidified atmosphere. Cimetidine and ranitidine were also dissolved directly in the culture medium. At the required time interval medium was removed and stored frozen for analysis. The cell monolayer was solubilized in aqueous NaOH (1 M) and the protein content determined [6]. To avoid confusion, the term culture or cultured is reserved in the text to refer to a period during which cells were in contact with WME containing no added substrate or inhibitors. In contrast, the term incubation is used to refer to a period during which cells were in contact with WME containing paracetamol and/or cimetidine and ranitidine.

Analysis of the conjugates of paracetamol was performed by HPLC [7]. Equipment consisted of Altex 110A pumps, 420 controller, Rheodyne 7125 injector, Waters μ-bondapak C-18 reverse phase column, Perkin–Elmer LC-15 detector and a Perkin–Elmer 56 pen recorder linked to a LDC 308 computing integrator. Paracetamol and conjugates were quantified by u.v. detection [7]; the validity of this method was confirmed by the use of [^{14}C]-paracetamol as substrate and collection of relevant peaks for scintillation counting.

Covalent binding of paracetamol (*p*-hydroxy[ring-3,5-^{14}C]-acetanilide) was measured essentially as described by Devalia *et al.* [8] except that hepatocytes were cultured on 100 mm petri dishes (Lux) coated with collagen. [3,5-^{14}C]-paracetamol was purified immediately before use by the method of Devalia and McLean [9]. Incubations were performed in a final volume of 5 ml. At the end of the incubation period medium was removed and stored for analysis and the cell monolayer washed five times with saline before removing the cells from the plate and proceeding with the solvent washing.

Estimates of lactic dehydrogenase activity were performed by measuring the disappearance of β-NADH at 340 nm in a Hewlett Packard 8450A spectrophotometer at ambient temperature. The incubation was carried out in HEPES buffer (pH 7.4) containing β-NADH (0.1 mM) and pyruvate (6.7 mM, final volume 3 ml). Enzyme activity in the culture medium was measured directly; activity in the rat hepatocyte monolayer was measured by the addition of triton X-100 (final concentration 0.2%) to the culture medium and waiting 10 min for complete cell lysis.

Measurement of protein synthesis was performed by the addition of L-[^{14}C]-leucine (0.5 μCi) to a plate of rat hepatocytes (35 mm well, final volume 2 ml). After 1 hr at 37° medium was removed and the monolayer washed 6 times with ice-cold saline (2 ml) containing leucine (1 mg/ml). Cells were then scraped from the plastic well and transferred into a plastic tube and cell protein precipitated by the addition of TCA. The precipitate was then centrifuged and washed twice with TCA (5%) before solubilising in NaOH (1 M). Radioactivity was determined by scintillation counting.

Treatment of results. The Michaelis–Menten kinetic parameters, K_m^{app} and V_{max}^{app} were determined by a rectangular hyperbolae iterative fit programme from Wilkinson [10]. Where applicable, results were analysed for statistical similarity by analysis of variance and the Students *t*-test (Minitab computing system). The computer programme RMICH2 [11] was used in the case of competitive inhibition.

RESULTS

Metabolism of paracetamol

Table 1 shows the effect of duration in culture on the formation of glucuronide, sulphate and glutathione conjugates of paracetamol by cultured rat hepatocytes. The rates of glucuronide and sulphate conjugate formation remained high in cells maintained in culture for 24 or 48 hr; the rate of glucuronide formation actually increasing at 48 hr compared to the earlier time-points. In contrast, the rate of glutathione conjugate formation was decreased even after 24 hr in culture but was not further reduced at 48 hr. The extent of covalent binding to cellular macromolecules was similarly decreased as culture time was increased.

Figure 1(a) shows the accumulation of paracetamol sulphate in the incubation medium at 0.5 mM paracetamol in rat hepatocytes maintained in culture for 48 hr and also in cultures of rat hepatocytes containing cimetidine (1 mM) and ranitidine (1 mM) added to the incubation medium. The accumulation of paracetamol sulphate was essentially linear over the entire incubation period of 24 hr and was unchanged by the inclusion of either cimetidine or ranitidine in the culture medium. During the period of incubation about 25% of the paracetamol was converted into conjugate. Figure 1(b)

Table 1. The effect of duration of culture on paracetamol metabolism by cultured rat hepatocytes

Time in culture (hr)	Paracetamol conjugate formation			Covalent binding
	Glucuronide	Sulphate (nmoles/mg protein/hr)	Glutathione	
2	19.9 ± 1.8	14.3 ± 1.1	3.6 ± 0.3	1.37 ± 0.10
24	23.0 ± 2.4	12.1 ± 1.2	1.3 ± 0.2	0.88 ± 0.12
48	38.2 ± 2.3	11.0 ± 0.7	1.5 ± 0.1	0.45 ± 0.04

Incubations were performed for a period of 6 hr as described in Methods. Paracetamol metabolism was measured by HPLC [7] and protein by the method of Lowry *et al.* [6]. Paracetamol concentration was 10 mM and the final amount of radioactivity was 2 μCi/plate. Results are expressed as mean ± 1 S.D. (N = 3).

shows that increasing the concentration of paracetamol to 5 mM resulted in virtually no increase in the rate of paracetamol sulphate formation, Moreover, cimetidine and ranitidine again failed to alter the accumulation of sulphate conjugate.

Figure 2(a) shows the accumulation of paracetamol glucuronide in the incubation medium at 0.5 mM paracetamol. The production of conjugate was linear during the incubation period but in this case both cimetidine and ranitidine caused a 50% inhibition of glucuronide accumulation in the medium. Despite the inhibition of glucuronide accumulation the linearity of conjugate formation remained throughout the incubation period. At higher paracetamol concentrations (Fig. 2b) the overall accumulation of glucuronide conjugate was much greater and the inhibitory effects of cimetidine and ranitidine decreased to about 75% of control values.

Figure 3 shows the effect of altering the concentration of cimetidine and ranitidine at a fixed concentration of paracetamol (1 mM). This concentration of paracetamol was chosen to approximate the apparent K_m of the glucuronidation pathway. As the concentration of either cimetidine or ranitidine was increased, a pronounced decline in glucuronide conjugate was observed. In both cases the decline in glucuronide conjugate was significant ($P < 0.01$). Again no change in sulphate conjugate accumulation was observed.

In order to obtain a clearer understanding of the potency with which these compounds inhibit glucuronidation, and also gain some insight into a mechanism of action, the effects of various concentrations of ranitidine on the apparent Michaelis–Menten parameters, V_{max}^{app} and K_m^{app}, for paracetamol glucuronidation were investigated. Hepatocytes were again cultured for 48 hr prior to incubation with paracetamol. Figure 4 shows the plot of paracetamol concentration against glucuronide formation during a 6 hr incubation period in the presence of 0.25, 1.0 and 2.0 mM ranitidine. The K_m^{app} for glucuronidation

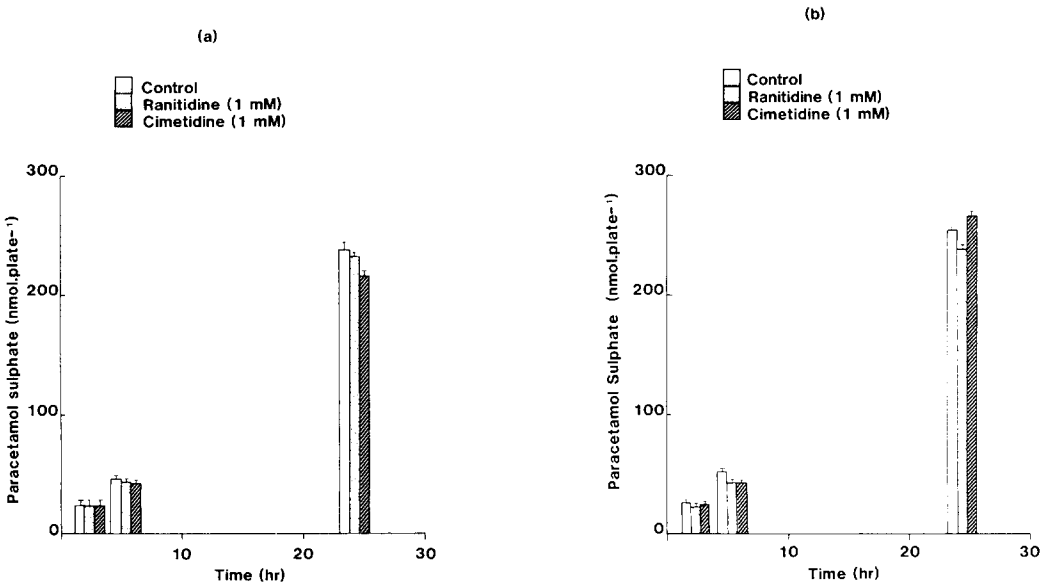

Fig. 1. The effect of cimetidine and ranitidine on paracetamol sulphate accumulation. Hepatocytes were cultured 48 hr before incubating the cells with paracetamol (0.5 mM, a; 5.0 mM, b) in the presence and absence of cimetidine and ranitidine. Paracetamol sulphate was analysed by HPLC [7] in triplicate and protein by the method of Lowry *et al.* [6] (mean ± S.D.; N = 15; 0.65 ± 0.05 mg/plate).

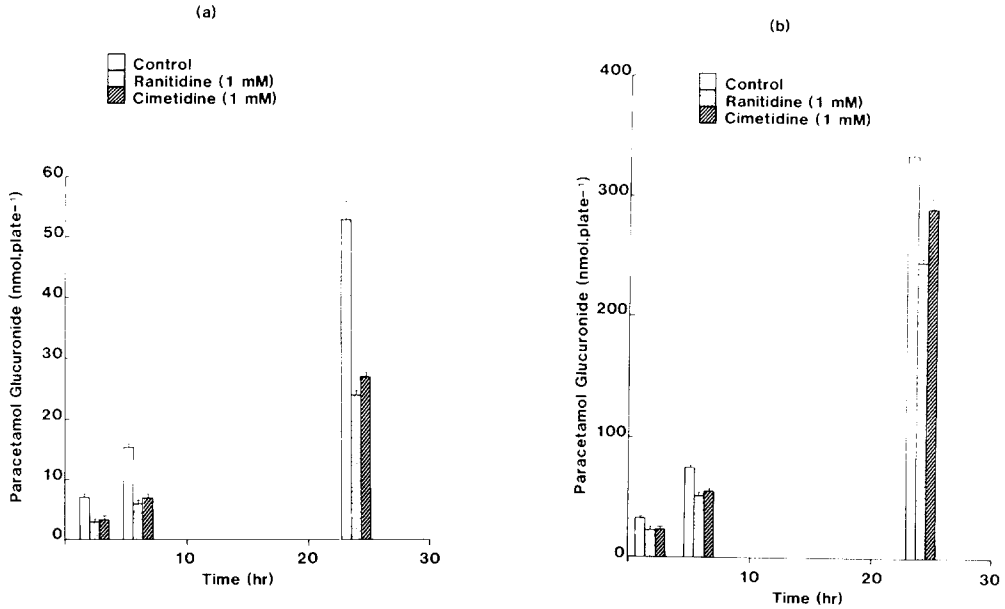

Fig. 2. The effect of cimetidine and ranitidine on paracetamol glucuronide accumulation. Hepatocytes were cultured 48 hr before incubating the cells with paracetamol (0.5 mM, a; 5.0 mM, b) in the presence and absence of cimetidine and ranitidine. Paracetamol glucuronide was analysed by HPLC [7] in triplicate. Protein was estimated by the method of Lowry *et al.* [6] (mean ± S.D.; N = 18; 0.65 ± 0.05 mg/plate).

of paracetamol was calculated to be 1050 ± 330 μM and the V_{max}^{app} for the reaction was 21.5 ± 2.0 nmoles/mg protein/hr (Table 2). At 0.25 mM ranitidine the K_m^{app} was increased 3-fold without any change in V_{max}^{app}, but at higher concentrations of ranitidine small decreases in V_{max}^{app} were observed. However, Lineweaver–Burk plots were linear at all concentrations of ranitidine.

Cell viability estimates

To assess the extent to which cimetidine or ranitidine may cause cell toxicity under our experimental conditions both lactic dehydrogenase activity and protein synthesis were monitored in cultured rat hepatocytes incubated with ranitidine and cimetidine. Neither cimetidine nor ranitidine (1 mM) caused leakage of lactic dehydrogenase into the culture medium in excess of that observed with control cultures, throughout a 24-hr incubation period (Table 3). Neither compound appeared to have significant effects on the incorporation of L-[^{14}C]-leucine into hepatocyte protein (Table 4).

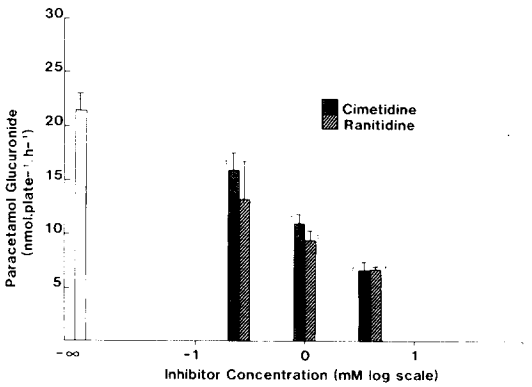

Fig. 3. The effect of various concentrations of either cimetidine or ranitidine on paracetamol glucuronide accumulation. Hepatocytes were cultured for 48 h before incubating with paracetamol (1 mM) in the presence or absence of various concentrations of cimetidine or ranitidine. Paracetamol glucuronide was measured by HPLC [7] in triplicate except control where N = 6 and protein by the method of Lowry *et al.* [6] (mean ± S.D.; N = 24; 0.73 ± 0.04 mg/plate). *P < 0.01 compared to control.

DISCUSSION

The choice of cultured hepatocytes as a model system to investigate potential effects of cimetidine and ranitidine on conjugation reaction was made on the assumption that cofactor levels and compartmentalization are important controlling factors in conjugation reactions. Thus selective inhibition of sulphation of paracetamol by hepatocytes can be achieved by removing extracellular sulphate whereas selective inhibition of glucuronidation can be achieved by preincubation with galactosamine (2 mM) with no observable effect on sulphate conjugation [4]. As can be seen in our present study rates of paracetamol sulphation and glucuronidation are well maintained in hepatocytes cultured for 48 hr, and the rate of glucuronidation increased in 48 hr cultured cells compared to hepatocytes maintained in culture for only 2 hr.

In contrast, the rate of glutathione conjugate formation was decreased in hepatocytes maintained in culture for 24 or 48 hr. This is consistent with the well known decrease in cytochrome P450 levels in rat hepatocytes maintained in culture [12]. In our

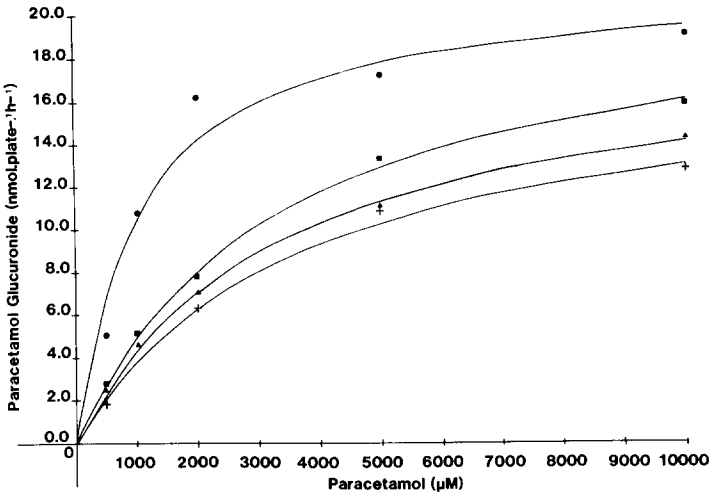

Fig. 4. The effect of ranitidine on the Michaelis–Menten kinetic parameters for paracetamol glucuronide formation. Hepatocytes were cultured for 48 hr before incubating with paracetamol in the presence of various concentrations of ranitidine (●, 0 mM; ■, 0.25 mM; ▲, 1.0 mM; ×, 2.0 mM). Incubation time 5.5 hr. Protein was estimated by the method of Lowry *et al.* [6] (mean ± S.D.; N = 60; 1.01 ± 0.05 mg/plate).

Table 2. The effect of ranitidine on the apparent K_m and V_{max} for paracetamol glucuronidation

Ranitidine (mM)	K_m^{app} (μM)	V_{max}^{app} (nmoles/mg protein/hr)
0	1052 ± 332	21.5 ± 2.0
0.25	3328 ± 285	21.4 ± 0.8
1.00	3391 ± 244	18.9 ± 0.6
2.00	3729 ± 616	17.9 ± 1.3

Hepatocytes were incubated with various concentrations of paracetamol (0.5, 1.0, 2.0, 5.0 and 10 mM) for a period of 6 hr as described in Methods and the glucuronide conjugate of paracetamol measured by HPLC [7]. Estimates were made in triplicate and K_m^{app} and V_{max}^{app} obtained by the method of Wilkinson [10].

experimental protocols we failed to observe any toxicity of paracetamol to the cultured rat hepatocytes and this we have attributed to two observations. The rat is known to be relatively insensitive to the toxic effects of paracetamol due to an unusually high K_m (paracetamol) value for the formation of the reactive metabolite [13]. The loss of cytochrome P450 from the cultured rat hepatocytes will further decrease the generation of active metabolite within the cell. In our protocols we have measured both the generation of the glutathione conjugate of paracetamol and the covalent binding of the reactive metabolite of paracetamol to cellular protein [14]. Covalent binding of paracetamol was reduced to about 33% of control values after 48 hr in culture. Thus, under the experimental conditions utilized paracetamol was non-toxic to the cultured hepatocytes (data not shown).

The inhibition of glucuronidation observed in this system by cimetidine and ranitidine appeared to be a relatively specific phenomena because of the negligible effects observed on sulphation. To assess the extent to which cimetidine or ranitidine may cause cell toxicity under these conditions, both LDH leakage and protein synthesis were monitored in rat

Table 3. The effect of cimetidine and ranitidine on LDH leakage

Time (hr)	LDH (% release) 2	4	24
Control (N = 4)	3.2 ± 0.8	7.7 ± 2.4	19.4 ± 1.1
Ranitidine (1 mM)	4.20 (2.3–6.1)	4.15 (4.1–4.2)	24.80 (23.1–26.5)
Cimetidine (1 mM)	2.30 (1.9–2.7)	5.00 (4.3–5.7)	19.00 (18.1–19.9)

Hepatocytes were prepared and incubations were performed as described in Methods. Data is presented as mean ± S.D. for control incubations and mean (range) for drug treatments.

Table 4. The effect of cimetidine and ranitidine on the incorporation of L-[^{14}C]-leucine into hepatocyte protein

Concentration (μM)	Protein synthesis (dpm/plate) Cimetidine	Ranitidine
0	5681 ± 630	
100	5056 ± 207	3526 ± 234
1000	4176 ± 459	4845 ± 350
5000	4605 ± 271	4502 ± 196

Hepatocytes were isolated and cultured for 48 hr as described in Methods. After 3 hr incubation with fresh medium 0.5 μCi L-[^{14}]-leucine was added to each plate. Incorporation of radioactivity was determined 1 hr later in triplicate as described in Methods. Results are expressed as mean ± 1 S.D. Total protein per plate was 0.73 ± 0.16 μg/plate (mean ± S.D., N = 21).

hepatocytes cultured with the histamine H_2-receptor antagonists. Neither cimetidine nor ranitidine (1 mM) caused leakage of LDH into the culture medium in excess of that observed with control cultures. In addition both compounds appeared to have little effect on the incorporation of L-[^{14}C]-leucine into hepatocyte protein. In our experiments, incorporation of L-[^{14}C]-leucine was measured over a period of 1 hr. We had previously established that incorporation of radiolabel into macromolecules was linear for at least 4 hr indicating that our incorporation experiments were a valid index of protein synthesis.

Only a limited amount of information is available concerning the effects of histamine H_2-receptor antagonists on conjugation reactions. In rat liver microsomes cimetidine and ranitidine failed to influence the glucuronidation of morphine [15] and paracetamol glucuronidation was shown to be unaffected by cimetidine [16]. In the clinic, cimetidine failed to alter the elimination of either lorazepam or oxazepam, both of which are eliminated exclusively by glucuronide conjugate formation [17]. In contrast, however, mean peak plasma levels of lorazepam were increased by cimetidine [18].

This literature data would suggest that a direct action of either cimetidine or ranitidine with the enzyme protein may not explain the inhibition of glucuronidation observed in our experiments but rather it may reflect changes in cofactor levels, enzyme latency or the function of cellular transport proteins. However, more recently, Mitchell *et al.* [19] have demonstrated that in addition to the effects of cimetidine on paracetamol oxidation, inhibition of paracetamol glucuronidation by cimetidine was observed in microsomes prepared from both rat and human liver. The inhibiting constant, *Ki*, for cimetidine in the inhibition of glucuronidation was $1390 \pm 230\ \mu$M, and this must be attributed to a direct effect with the transferase protein(s). In studies of the effects of cimetidine upon the pharmacokinetics of paracetamol in normal volunteers, these authors showed that cimetidine reduced mean plasma clearance of paracetamol, decreased the fractional clearance by oxidation and glucuronidation but had no effect on the fractional clearance by sulphation. In this respect it is of interest that a polar conjugate of cimetidine had been tentatively identified as cimetidine *N*-glucuronide in man [20] and thus a competitive inhibition of other glucuronidation reactions may be expected. Thus, our studies with cultured rat hepatocytes clearly support the experimental findings obtained by Mitchell *et al.* [19] concerning the effects of cimetidine on paracetamol conjugation reactions.

Previous studies have shown that cimetidine binds much more avidly to cytochrome P450 than does ranitidine [1–3]. However, cimetidine and ranitidine appear to have similar potency when considering the inhibition of glucuronidation suggesting that different structural features may be responsible for the interaction of the histamine H_2-receptor antagonists with each enzyme system. The inhibition constant *Ki*, can be calculated for the inhibition of both cytochrome P450 and glucuronidation pathways in rat hepatic microsomes. Mitchell *et al.* [19] calculated a value of $130 \pm 16\ \mu$M for the inhibition of paracetamol–cysteine adduct formation assuming a mixed but primarily competitive type of inhibition. The *Ki* value for inhibition of glucuronidation by cimetidine was $1390 \pm 230\ \mu$M assuming a competitive type of inhibition.

In the case of ranitidine a more detailed kinetic investigation was undertaken in our hepatocyte model (Fig. 4). At 0.25 mM ranitidine, the inhibition appeared to be competitive whereas small decreases in V_{max}^{app} were apparent at 1.0 and 2.0 mM ranitidine. If the mechanism of inhibition is assumed to be purely competitive then a *Ki* value of $435 \pm 134\ \mu$M can be calculated for ranitidine. However, the Dixon plot for this experiment may be interpreted as nonlinear and as a consequence a further secondary plot was performed.

This was a plot of reciprocal fractional inhibition $(V - Vi)/V$ against reciprocal inhibitor concentration. This plot was consistent with partial competitive inhibition with an estimated *Ki* of 48–160 μM. These estimates of *Ki* would suggest that the inhibition of glucuronidation by ranitidine may be observable *in vivo*. The consequences of inhibiting the glucuronidation of paracetamol may well be important in that drug could then be diverted into the toxic cytochrome P450 pathway [14]. However, in the case of cimetidine, protection against paracetamol hepatotoxicity has been reported in rats [16, 19]; the authors suggesting that cimetidine inhibited the formation of the reactive metabolite of paracetamol both *in vivo* and *in vitro*. In contrast, ranitidine is much less potent at inhibiting the cytochrome P450 system and as such may potentiate the toxicity of paracetamol when the analgesic is taken in overdose and in accidental poisoning [21].

In conclusion, these studies indicate a potential for both cimetidine and ranitidine to inhibit the glucuronidation of paracetamol *in vivo*. Clearly, the exact meaning of apparent K_m and V_{max} values must be interpreted with caution in intact cellular systems where an enzymic reaction may have several rate-limiting factors. However, our results do provide an explanation for the findings of Leonard and Dent [22] who noted a marked potentiation of paracetamol-induced hepatotoxicity in the rat by ranitidine but not cimetidine. Further studies are required to assess the importance of these findings in an appropriate animal model and in the clinic.

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