MTS and Pitot HC, Use of a low-speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. *In Vitro* **22**: 201–211, 1986.
19. Stacey NH, Effects of chlorpromazine on taurocholate transport in isolated rat hepatocytes. *Biochem Pharmacol* **37**: 4129–4134, 1988.
20. Eaton DL and Klaassen CD, Carrier-mediated transport of ouabain in isolated hepatocytes. *J Pharmacol Exp Ther* **205**: 480–488, 1978.
21. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
22. Le Thei B, Dumont M, Michel A, Erlinger S and Houssin D, Cholestatic effect of cyclosporine in the rat, an inhibition of bile acid secretion. *Transplantation* **46**: 510–512, 1988.
23. Gulbis B, Adler M, Ooms HA, Desmet JM, Leclerc JL and Primo G, Liver-function studies in heart-transplant recipients treated with cyclosporin A. *Clin Chem* **34**: 1772–1774, 1988.
24. Ried M, Gibbons S, Kwok D, Van Buren CT, Flechner S and Kahan BD, Cyclosporine levels in human tissues of patients treated for one week to one year. *Transplant Proc* **15**(Suppl 1): 2434–2437, 1983.
25. Ziegler K, Frimmer M and Koepsell H, Photoaffinity labeling of membrane proteins from rat liver and pig kidney with cyclosporine diazirine. *Transplantation* **46**: 15s–20s, 1988.
26. Boelsterli UA, Bouis P, Brouillard JF and Donatsch P, *In vitro* toxicity assessment of cyclosporin A and its analogs in a primary rat hepatocyte culture model. *Toxicol Appl Pharmacol* **96**: 212–221, 1988.
27. Ananthanarayanan M, Von Dippe P and Levy D, Identification of the hepatocyte Na^+-dependent bile acid transport protein using monoclonal antibodies. *J Biol Chem* **263**: 8338–8343, 1988.
28. Frimmer M and Ziegler K, The transport of bile acids in liver cells. *Biochim Biophys Acta* **947**: 75–99, 1988.
29. Kukongviriyapan V, Kotecka B and Stacey NH, Substrate transport and cyclosporin A in isolated rat hepatocytes. *Biochem Pharmacol* **42**: 193–195, 1991.

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In vitro effect of clofibric acid derivatives on rat hepatic microsomal electron transport chains

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Clofibric acid (CFB*), bezafibrate (BZB) and gemfibrozil (GFB) are structurally related drugs widely used in the treatment of hyperlipoproteinemias [1–3]. Their administration to both experimental animals and humans induces a multiplicity of physiological changes: hepatomegaly, smooth endoplasmic reticulum and cytochrome P450 induction, peroxisomal proliferation [4, 5], inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase and very low density lipoprotein synthesis [6]. Nevertheless, there is no agreement about the relative contribution of the mechanisms responsible for the hypolipidemic effect.

Rat liver microsomes possess two electron transport chains [7] (Scheme 1): one of them, the microsomal NADH–cytochrome *c* reductase system, consists of cytochrome b_5 and its flavoprotein, NADH–cytochrome b_5 reductase (EC 1.6.2.2). The second system utilizes NADPH, and the electrons flow from the NADPH–cytochrome *c* reductase (EC 1.6.2.4) to cytochrome P450, and also to cytochrome b_5. Reducing equivalents flowing through cytochrome b_5 are involved in important lipid biotransformation reactions such as fatty acid chain elongation and desaturation [8]. Also, isoenzymes of cytochrome P450 are involved in ω-hydroxylation of long chain fatty acids [5].

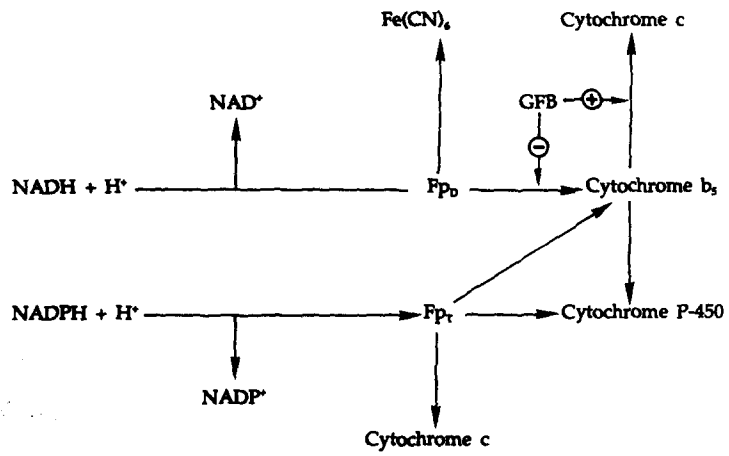
The quality of fatty acids (chain length and number of unsaturations), either in the free form or incorporated into glycerolipids, plays an important role controlling lipoprotein metabolism [9, 10]. Given that the hypolipidemic effect of clofibric acid derivatives is not well understood, we have been interested in testing the effect of these drugs on enzymes related to the hepatic fatty acid synthesis, either *in vitro* or *in vivo*. As part of this project, we have studied the *in vitro* effect of CFB, BZB and GFB on the two microsomal electron transport chains.

Material and Methods

NADH, NADPH, cytochrome *c* (Type III from horse heart), CFB and Trizma base (Tris[hydroxymethyl] aminomethane) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.); BZB and GFB were a generous gift from Lab. Andreu (Barcelona, Spain) and Lab. Parke-Davis S.A. (Barcelona, Spain), respectively. PMSF was from Boehringer Mannheim (Mannheim, Germany), EDTA from Merck (Darmstadt, Germany), and dithiothreitol from Fluka (Buchs, Switzerland). Other general chemicals were obtained from commercial sources and were of the highest purity available.

Microsomes were prepared from the livers of Sprague–Dawley male rats (200–220 g) aged 7–8 weeks. Following 18 hr of starvation, the animals were killed by decapitation between 8 and 9 a.m. Livers were perfused with ice-cold 0.9% NaCl. The tissue was homogenized in 0.2 mM EDTA, 0.1 mM PMSF, 50 mM NaF, 0.25 M sucrose, 50 mM Tris–HCl buffer, pH 7.4, and microsomes were obtained as described previously [11], and resuspended in 0.1 M phosphate buffer pH 7.4. Protein concentration was determined by the method of Bradford [12], using bovine serum albumin as standard. NADH–cytochrome *c* reductase activity was determined by the method of Yasukochi and Masters [13]. The assay mixture contained, in final concentrations, 0.3 mM KCN, 50 μM cytochrome *c*, 100 μM EDTA, 0.1 M phosphate buffer pH 7.4, and microsomal

* Abbreviations: CFB, clofibric acid; BZB, bezafibrate; GFB, gemfibrozil; PMSF, phenylmethylsuphonylfluoride.



Scheme 1. Hepatic microsomal electron transport pathways. Fp_d = NADH–cytochrome b_5 reductase. Fp_T = NADPH–cytochrome *c* reductase. GFB = gemfibrozil. NADH–cytochrome c reductase activity involves the flow of electrons from NADH to Fp_D to cytochrome b_5, and then to cytochrome *c*.

protein at varying concentrations. After 5 min preincubation at 37°, the reaction was started by adding 120 μM NADH, and the absorbance increase at 550 nm was recorded on a Perkin–Elmer 550S UV-Vis spectrophotometer with a recorder accessory. The rate of reduction of cytochrome *c* was measured using an extinction coefficient of 18.5 $mM^{-1}\,cm^{-1}$. NADPH–cytochrome *c* reductase activity was measured by the same method, but using NADPH instead of NADH as electron donor. For measuring NADH–cytochrome b_5 reductase activity, a reaction mixture including 0.25 mM $K_3Fe(CN)_6$, 0.1 M phosphate buffer, pH 7.4, and microsomal protein at varying concentrations was used [14]. NADH (0.15 mM) was added after 5 min of preincubation at room temperature, and the absorbance change at 420 nm was recorded. The rate of reduction of potassium ferricyanide was measured using an extinction coefficient of 1.02 $mM^{-1}\,cm^{-1}$. When required, drugs were added to the incubation medium from stock solutions adjusted at pH between 7.5 and 8 with 0.1 N NaOH. The volume added did not modify the pH of the assay mixture.

For the dialysis assay, a Spectrapor No. 3 membrane tubing (Spectrum Medical Inds., Los Angeles, CA, U.S.A.) was used, dialysing at 4° against 2 × 1 L of 1 mM dithiothreitol, 0.1 M phosphate buffer, pH 7.4, for 24 hr.

All kinetic calculations were done using the Enzfitter data analysis program.

Results and Discussion

The effect of CFB, BZB and GFB, at two different concentrations (1 and 5 mM) on the activities of microsomal electron transport chain enzymes, namely NADH–cytochrome *c* reductase, NADH–cytochrome b_5 reductase, and NADPH–cytochrome *c* reductase, was investigated. As shown in Table 1, the only effect is the stimulation of NADH–cytochrome *c* reductase activity by the three drugs, resembling their relative order of potency (GFB > BZB > CFB), observed in relation to the therapeutic activity [1–3]. Neither NADPH–cytochrome *c* reductase nor NADH–cytochrome b_5 reductase activities were markedly affected by the additon of CFB, BZB or GFB.

The stimulation of NADH–cytochrome *c* reductase by GFB seemed not to be an artefact, as no change in

Table 1. *In vitro* effect of CFB, BZB and GFB on rat hepatic microsomal NADH–cytochrome *c* reductase, NADPH–cytochrome *c* reductase and NADH–cytochrome b_5 reductase activities

	NADH–cytochrome *c* reductase (%)	NADPH–cytochrome *c* reductase (%)	NADH-cytochrome b_5 reductase (%)
CFB 1 mM	109	107	98
CFB 5 mM	121	108	108
BZB 1 mM	114	110	103
BZB 5 mM	127	108	108
GFB 1 mM	118	100	95
GFB 5 mM	186	115	100

Results are expressed as per cent activities with respect to controls (100% = 1417 nmol/min/mg microsomal protein for NADH–cytochrome *c* reductase, 100% = 327 nmol/min/mg for NADPH–cytochrome *c* reductase, 100% = 6.4 μmol/min/mg for NADH–cytochrome b_5 reductase) with no drug addition. Values are the mean of two experiments performed in duplicate, with microsomes from two pooled rat livers. Enzymatic activities were assayed as described in Materials and Methods.

absorbance was detected when the drug, cytochrome *c* and NADH were incubated together in the absence of microsomes.

GFB produced a concentration-dependent increase in NADH–cytochrome *c* reductase activity, as can be seen in Table 2. All the values were obtained from microsomes preincubated for 5 min in the presence of the stated drug concentration; no difference in stimulation effectivity was found with changing preincubation times, from 0 to 15 min (data not shown). To be able to obtain the theoretical maximum effect, a Scatchard analysis of these results was done [15]. Plotting % stimulation/mM GFB versus % stimulation, a regression line with a correlation coefficient of -0.904 ($P < 0.05$) was obtained. E_{max} was determined from the *x*-intercept of the regression line, with a value of 295% of stimulation (giving 0% stimulation to control values). The EC_{50} was calculated from the abscissa coordinate corresponding to $E_{max}/2\%$ response, giving a value of 9.5 mM. Although this value is higher than the peak plasma concentration in humans after therapeutic dosages (0.1–0.8 mM) [16], the concentrations needed to obtain a 50% stimulation are very close to this range. Also, knowing the ability of GFB to concentrate in liver and kidney [3], an *in vivo* effect should not be discarded. Only Ivanetich *et al.* [17] have reported a similar effect, showing an *in vitro* stimulation of NADH–cytochrome *c* reductase activity (41% over control values) by adding an anesthetic agent (enflurane 14 mM) to the incubation medium. No attempt was made to elucidate these results further.

The influence of microsomal protein concentration on the stimulation of NADH–cytochrome *c* reductase activity by GFB was studied. No significant variation in the amount of stimulation was found over the whole range of microsomal protein tested (25–150 μg/mL of incubation medium).

To determine whether the effect of GFB on NADH–cytochrome *c* reductase activity was reversible, we attempted to eliminate the drug from the assay medium. The stimulation persisted even after two centrifugations at 105,000 *g* for 30 min, although it disappeared when the assay medium was dialysed for 24 hr (46% stimulation before—5% after dialysis, using 2 mM GFB).

In order to study the kinetics of the stimulation, the NADH–cytochrome *c* reductase assay was carried out at different cytochrome *c* concentrations in the presence of 2 and 5 mM of GFB and fixed NADH concentrations. Eadie–Hofstee plots of the results provided the apparent kinetic constants showed in Table 3A. GFB addition did not modify the apparent K_m value of cytochrome *c* (no significant differences with parallel lines test), but it did induce an increase in apparent V_{max} values, from 1500 nmol/min/mg in control to 2330 and 2960 nmol/min/mg in the presence of 2 and 5 mM GFB, respectively.

When cytochrome *c* concentration was held constant and NADH concentration was changed, a different picture emerged (Table 3B). GFB addition produced an increase in the apparent V_{max} values, but the addition of 5 mM GFB also led to a significant increase in the apparent K_m value with respect to the control ($P < 0.05$, parallel lines test).

The hepatic microsomal electron transport chains are shown in Scheme 1. From the results, it seems reasonable to postulate that GFB interacts with the electron flow through cytochrome b_5. With changing concentrations of cytochrome *c* there is a marked increase in apparent V_{max} with no modification of apparent K_m values. Thus, GFB seems to facilitate the transfer of electrons from reduced cytochrome b_5 to the artificial acceptor, cytochrome *c*. When cytochrome *c* concentration is fixed and NADH concentration varies, the overall situation is different, GFB producing a stimulation of NADH–cytochrome *c* reductase activity by increasing the apparent V_{max} of the reaction. However, there is a concomitant decrease in NADH affinity, shown by the elevation of the apparent K_m values,

Table 2. Concentration dependent stimulation of NADH–cytochrome *c* reductase activity by GFB

GFB (mM)	Activity*	% Stimulation
0	800 ± 53	0
0.5	957 ± 118	20
1	1033 ± 99	30
1.5	1147 ± 121	43
2.5	1251 ± 83	56
5	1605 ± 56	100

* Values (nmol/min/mg microsomal protein) are means ± SEM from three different experiments performed in duplicate. For each experiment, microsomes were obtained from two pooled rat livers.

Table 3. Apparent cytochrome *c* and NADH kinetic constants from Eadie–Hofstee plots: effect of GFB addition

	Control	2 mM GFB	5 mM GFB
(A) Apparent cytochrome *c* kinetic constants, [NADH] = 120 μM			
K_m*	18.4	21.7	19.3
V_{max}†	1500	2330	2960
(B) Apparent NADH kinetic constants, [cytochrome *c*] = 50 μM			
K_m*	5.9	7.4	10.9
V_{max}†	1130	1470	1670

* μM.
† nmol/min/mg microsomal protein.

at least in the presence of 5 mM GFB. Indeed, at the lowest NADH concentrations tested (2.5, 5 μM), the presence of 5 mM GFB did not modify the NADH–cytochrome *c* reductase activity but rather produced a slight inhibition. Given that NADH–cytochrome b_5 reductase activity is not affected by GFB addition at the concentrations used, the drug seems to hinder the flow of electrons from the flavoprotein to the cytochrome b_5, although in the presence of cytochrome *c* this difficulty is overcome by the increase in electron flow from the hemoprotein to the artificial acceptor. Moreover, it would not be reasonable to ascribe the increase in electron flow to cytochrome *c* to a modification of the cytochrome *c* itself, given that NADPH–cytochrome *c* reductase activity, where electrons flow directly from the flavo- to the hemoprotein, is not affected by GFB addition.

In vivo effects of clofibric acid derivatives on the components of hepatic microsomal electron transport chains have only been studied with CFB, showing a completely different behaviour, decreasing the NADH–cytochrome *c* reductase activity after the rats have been fed a diet containing 0.5% (w/w) of the drug, with no alteration of NADH–cytochrome b_5 or cytochrome b_5 levels [18]. Work is being done now in our laboratory to determine the *in vivo* effect of clofibric acid derivatives on the hepatic microsomal electron transport chains, in order to establish a possible relationship between the *in vitro* and *in vivo* results.

In summary, the *in vitro* effect of clofibric acid derivatives (clofibric acid, bezafibrate and gemfibrozil) on rat hepatic microsomal NADPH–cytochrome *c* reductase, NADH–cytochrome b_5 reductase and NADH–cytochrome *c* reductase has been studied. GFB produces a marked concentration-related stimulation of NADH–cytochrome *c* reductase activity, showing an EC_{50} of 9.5 mM and an E_{max}

of 295% stimulation (0% control values). Kinetic data obtained in the presence of gemfibrozil indicate that this drug could act by enhancing the electron flow from cytochrome b_5 to cytochrome *c* and by inhibiting the electron flow from NADH to cytochrome b_5.

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REFERENCES

1. Dujovne CA and Harris WS, The pharmacological treatment of dyslipidemia. *Annu Rev Pharmacol Toxicol* **29**: 265–288, 1989.
2. Monk JP and Todd PA, Bezafibrate. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic uses in hyperlipidaemia. *Drugs* **33**: 539–576, 1987.
3. Todd PE and Ward A, Gemfibrozil: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic use in dyslipidaemia. *Drugs* **36**: 314–339, 1988.
4. Hawkins JM, Jones WE, Bonner FW and Gibson GG, The effect of peroxisome proliferators on microsomal, peroxisomal, and mitochondrial enzyme activities in the liver and kidney. *Drug Metab Rev* **18**: 441–515, 1987.
5. Sharma R, Lake BG, Foster J and Gibson GG, Microsomal cytochrome P-452 induction and peroxisome proliferation by hipolipidaemic agents in rat liver. A mechanistic interrelationship. *Biochem Pharmacol* **37**: 1193–1201, 1988.
6. Shepherd J and Packard CJ, Pharmacological approaches to the modulation of plasma cholesterol. *Trends Pharmacol Sci* **9**: 326–329, 1988.
7. Eriksson LC, DePierre JW and Dallner G, Preparation and properties of microsomal fractions. In: *Hepatic Cytochrome P-450 Monooxygenase System* (Ed. Schenkman JB and Kupfer D), pp. 9–39. Pergamon Press, New York, 1982.
8. Nagi M, Cook L, Prasad MR and Cinti DL, Site of participation of cytochrome b_5 in hepatic microsomal fatty acid chain elongation. Electron input in the first reduction step. *J Biol Chem* **258**: 14823–14828, 1983.
9. Bihain BE, Deckelbaum RJ, Yen FT, Gleeson AM, Carpentier YA and Witte LD, Unesterified fatty acids inhibit the binding of low density lipoproteins to the human fibroblast low density lipoprotein receptor. *J Biol Chem* **264**: 17316–17321, 1989.
10. Desreumaux C, Dedonder E, Dewailly P, Sezille G and Fruchart JC, Effects of unsaturated fatty acids in phospholipids on the *in vitro* activation of the lipoprotein lipase and the tryglyceride lipase. *Arzneim Forsch/Drug Res* **29**: 1581–1583, 1979.
11. Nagi M, Laguna JC, Cook L and Cinti DL, Disruption of rat hepatic microsomal electron transport chains by the selenium-containing anti-inflammatory agent ebselen. *Arch Biochem Biophys* **269**: 264–271, 1989.
12. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
13. Yasukochi Y and Masters BSS, Some properties of a detergent-solubilized NADPH-cytochrome *c* reductase purified by biospecific affinity chromatography. *J Biol Chem* **251**: 5337–5344, 1976.
14. Strittmatter P, Cytochrome b_5. In: *Methods in Enzymology* (Eds. Estabrook RW and Pullman ME), Vol. 10, pp. 561–565. Academic Press, New York, 1967.
15. Kocarek TA and Feller DR, Quantitative assessment of enzyme induction by peroxisome proliferators and application to determination of effects on triglyceride biosynthesis in primary cultures of rat hepatocytes. *Biochem Pharmacol* **38**: 4169–4176, 1989.
16. Brandes R, Kaikus RM, Lysenko N, Ockner RK and Bass NM, Induction of fatty acid binding protein by peroxisome proliferators in primary hepatocyte cultures and its relationship to the induction of peroxisomal β-oxidation. *Biochim Biophys Acta* **1034**: 53–61, 1990.
17. Ivanetich KM, Manca V, Harrison GG and Berman M, Enflurane and methoxyflurane: their interaction with hepatic microsomal stearate desaturase. *Biochem Pharmacol* **29**: 27–34, 1980.
18. Kawashima Y, Uy-Yu N and Kozuka H, Sex-related differences in the enhancing effects of perfluorooctanoic acid on stearoyl-CoA desaturase and its influence on the acyl composition of phospholipid in rat liver. Comparison with clofibric acid and tiadenol. *Biochem J* **263**: 897–904, 1989.

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