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INDUCTION OF PEROXISOMAL FATTY ACYL-CoA OXIDASE AND MICROSOMAL LAURATE HYDROXYLASE ACTIVITIES BY BECLOBRIC ACID AND TWO METABOLITES IN PRIMARY CULTURES OF RAT HEPATOCYTES

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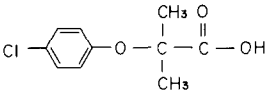
Abstract—Beclobrate [2-(4-[(4-chlorophenyl)methyl]phenoxy)-2-methylbutyric acid ethyl ester], a structural analog of clofibrate, is used clinically as a lipid-lowering agent. Although, like clofibrate, beclobrate produces a profound hepatomegalic response in rodents, no studies of this drug on hepatic peroxisome proliferation have appeared. We have examined, relative to clofibric acid (CPIB), the concentration-dependent effects of beclobric acid (Beclo), the active moiety of beclobrate, and two oxidized metabolites [a carbinol (M2) and a benzophenone (M3)] on peroxisomal fatty acyl-CoA oxidase (FACO) and microsomal laurate hydroxylase (LH) activities in primary cultures of rat hepatocytes. All compounds induced FACO and LH activities in a concentration-dependent manner after a 72 hr incubation with the cultured cells. Beclo was 4.8- and 6.5-fold more potent than CPIB as an inducer of FACO and LH respectively. M2 and M3 were more potent than Beclo as inducers of FACO and LH. Additionally, all compounds produced significant elevations relative to untreated control cultures in cellular lactate dehydrogenase activity (1.6- to 2.2-fold). We conclude that (1) Beclo is more potent than CPIB as an inducer of peroxisome proliferation-associated enzyme activities; (2) two metabolites of Beclo are more potent than the parent molecule as inducers of these activities and (3) these metabolites may contribute to the lipid-lowering and/or hepatomegalic effects of beclobrate in rats.

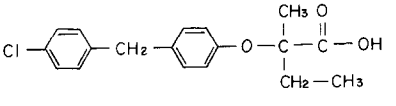
Clofibrate, structurally related (e.g. fenofibrate and nafenopin) and unrelated (e.g. Wy-14693 and BR-931) hypolipidemic agents, and certain plasticizers [e.g. di(2-ethylhexyl)phthalate] and herbicides (e.g. 2,4,5-trichlorophenoxyacetic acid) are members of a class of chemicals referred to as peroxisome proliferators [1, 4]. These compounds produce hepatomegaly, proliferation of hepatic peroxisomes, and induction of various peroxisomal and non-peroxisomal enzymes in rodents [1, 2]. Additionally, they comprise a class of non-mutagenic hepatocarcinogens [1, 2, 5]. The peroxisome proliferation-associated enzyme induction has been implicated in both the lipid-lowering [2, 6–8] and tumor-producing activities of these compounds in rodents [1, 2, 5].

Beclobrate [2-(4-[(4-chlorophenyl)methyl]phenoxy)-2-methylbutyric acid ethyl ester] is a new hypolipidemic agent which bears some structural resemblance to clofibrate (see Fig. 1). In rats, beclobrate is 22-fold more potent in its hepatomegalic effect than clofibrate and is 11- and 36-fold more potent than clofibrate as a hypocholesterolemic and hypotriglyceridemic agent respectively [9]. Like clofibrate, which is hydrolyzed *in vivo* by tissue and serum esterases to the active compound, clofibric acid (CPIB) [10], beclobrate is deesterified to its active form, beclobric acid (Beclo) [11]. Both CPIB and Beclo are then glucuronidated and excreted [10, 11].

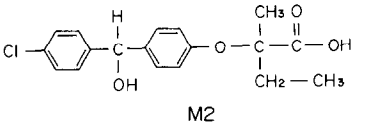
Recently, several phase I metabolites of Beclo have been isolated and identified [11]. These include two species, a carbinol (M2) and a benzophenone (M3), which are oxidized at the methylene bridge position of Beclo (see Fig. 1). These metabolites are



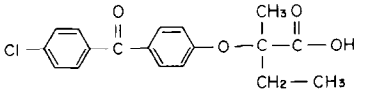
CLOFIBRIC ACID



BECLOBRIC ACID



M2



M3

Fig. 1. Chemical structures of clofibric acid (CPIB), beclobric acid (Beclo), and carbinol (M2) and benzophenone (M3), metabolites of beclobric acid.

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reported to be at least as active as Beclo in their hepatomegalic and hypolipidemic effects *in vivo*.*

The major objective of this study was to determine, relative to CPIB, the abilities of Beclo and its two oxidized metabolites (M2 and M3) to act as peroxisome proliferators in primary cultures of adult rat hepatocytes. This was to be inferred by measuring the activities of two enzymes known to undergo induction following treatment with peroxisome proliferating drugs; peroxisomal fatty acyl-CoA oxidase (FACO; the first and rate-limiting enzyme of the peroxisomal fatty acid β-oxidation system) [7, 12] and microsomal laurate hydroxylase (LH; the form(s) of cytochrome P-450 (P-452) which catalyzes the ω and ω-1 hydroxylations of lauric acid) [13]. A preliminary report of these results has appeared [14, 15].

MATERIALS AND METHODS

Materials. Biochemicals and their sources were: beclobric acid [2-(4-[(4-chlorophenyl)methyl]phenoxy)-2-methylbutyric acid; Beclo], M2 (carbinol metabolite of Beclo) and M3 (benzophenone metabolite of Beclo) (a gift from Dr. U. Jahn, Siegfried Ltd., Zofingen, Switzerland); collagenase Type IV (Cooper Biochemical, Malvern, PA); Nu-Serum (Collaborative Research Inc., Lexington, MA); Vitrogen (The Collagen Corp., Palo Alto, CA); Williams Medium E (Gibco, Grand Island, NY); [1-^{14}C]lauric acid (26 mCi/mmol) (Amersham, Arlington Heights, IL); and other biochemicals (Sigma Chemical Co., St. Louis, MO). Sources of other materials were: Corning tissue culture dishes (100 × 20 mm) and Spectra/Mesh (60, 111, and 202 μm) (Fisher Scientific, Cincinnati, OH) and Millex-GV filters (0.22 μm) (Millipore Corp., Medford, MA). Male Sprague–Dawley rats (150–275 g) were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN), housed in an animal facility accredited by the American Association for the Advancement of Laboratory Animal Care, and given food and water *ad lib.*

Primary culture of adult rat hepatocytes. Hepatocytes were isolated from male Sprague–Dawley rats by the recirculating collagenase perfusion method of Seglen [16]. Following liver perfusion, cells were collected in Williams Medium E containing 1% bovine serum albumin and filtered through 202 μm and 111 μm polyethylene Spectra/Mesh. The crude suspension was purified by centrifuging three times for 2 min at 50 *g* (the first two spins containing a few mg deoxyribonuclease I) followed by filtration through 60 μm nylon Spectra/Mesh. Cell yield and viability (greater than 90%) were determined by trypan blue exclusion cell counting. The hepatocyte suspension was then diluted with Williams Medium E supplemented with 10 μm hydrocortisone 21-hemisuccinate, 5 μM dexamethasone, 20 mIU/ml insulin, 100 μM 5-aminolevulinic acid, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.125 μg/ml amphotericin B, and 10% Nu-Serum and plated onto collagen-coated (Vitrogen) 100 mm petri dishes at a density of 2.5 million cells/5 ml medium. The dishes were placed in a humidified 37°, 95% air/5% CO_2 incubator, and cells were allowed 3 hr for attachment.

Drugs were dissolved directly in medium by sonication, and the resulting solutions were sterilized by filtration through 0.22 μm Millex-GV filters. After the attachment period, fresh medium containing drugs was added. Medium and drugs were subsequently renewed at 24 hr intervals. Using measurements of protein, DNA and lactate dehydrogenase (LDH) as criteria, CPIB, M2 and M3 were essentially noncytotoxic to the cultured hepatocytes at the highest concentrations (1, 0.3 and 0.3 mM respectively) used in these experiments. Beclo was noncytotoxic at concentrations up to 0.1 mM but was variably cytotoxic at 0.3 mM.

After 72 hr of incubation, dishes were washed twice with ice-cold 0.154 M KCl/50 mM Tris–HCl buffer, pH 7.4 (Tris–KCl), and hepatocytes were harvested by scraping into 1.25 ml Tris–KCl. The cells were homogenized by sonication, and aliquots of each homogenate were (1) taken for assay of LDH activity, (2) diluted 2 : 1 with Tris–KCl and frozen at −20° until assayed for FACO activity and (3) diluted 2 : 1 with 0.1 N NaOH and refrigerated until assayed for protein and DNA. The remaining quantity of each homogenate was frozen at −80° until assayed for LH activity.

Biochemical assays. Protein and DNA were determined by the methods of Lowry *et al.* [17] and Hinegardner [18] respectively. LDH activities in medium and sonicates were determined by following the pyruvate-dependent oxidation of NADH at 340 nm. FACO activity was assayed by the fluorometric method of Walusimbi-Kisitu and Harrison [19].

LH activity was assayed by measuring the conversion of [1-^{14}C]lauric acid to combined 11- and 12-hydroxylauric acids according to the thin-layer chromatographic method described by Lake *et al.* [20] with minor modifications. Incubations included 1–2 mg cellular protein and an NADPH-generating system consisting of 25 mM NADP, 500 mM glucose-6-phosphate, 25 U/ml glucose-6-phosphate dehydrogenase, and 313 mM $MgCl_2$. Lauric acid was introduced to the samples in 2 μl of 95% ethanol (0.1% of total volume).

Analysis of data. All culture dishes receiving a particular treatment (drug and concentration) were grouped together over all experiments for calculation of results (N = 3–37 dishes). Enzyme activities were expressed per mg cellular protein or mg (or μg) DNA as the mean ± SE. Comparisons among means were made at $P < 0.05$ using one-way analysis of variance and the Student–Newman–Keuls test.

The EC_{50} values for the induction of FACO and LH were determined in the following manner. Concentration–response data were plotted, and data points lying on the linear portions of the plots were subjected to a linear regression analysis for grouped data. CPIB, at 1 mM, was assumed to elicit a maximal inductive response in both assays. The EC_{50} values and 95% confidence intervals were calculated from the abscissa coordinates of the regression lines which corresponded to one-half the response to 1 mM CPIB. EC_{50} Values were considered to be

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significantly different if their 95% confidence intervals did not overlap.

RESULTS

Comparison of beclobric (Beclo) and clofibric (CPIB) acids on fatty acyl-CoA oxidase (FACO) and laurate hydroxylase (LH) activities in cultured rat hepatocytes. CPIB and Beclo each induced FACO and LH in a concentration-dependent manner when incubated with cultured hepatocytes for 72 hr (Fig. 2). Control FACO activity was 1.11 nmol $H_2O_2 \cdot min^{-1} \cdot (mg\ protein)^{-1}$ at this time, and increases of 11.3- and 8.6-fold were seen for 1 mM CPIB and 0.3 mM Beclo respectively. Control LH activity was 5.92 $nmol \cdot hr^{-1} \cdot (mg\ protein)^{-1}$ after 72 hr, and 1 mM CPIB and 0.3 mM Beclo caused increases in activity of 10.6- and 9.3-fold respectively. The EC_{50} (95% C.I.) values (mM) for FACO induction of 0.29 (0.20–0.42) and 0.061 (0.042–0.089) were determined for CPIB and Beclo, respectively, using the linear regression analysis described above. These values were significantly different by non-overlap of the 95% confidence intervals. The EC_{50} (95% C.I.) values (mM) for LH induction of 0.30 (0.19–0.46) and 0.046 (0.032–0.065) were determined for CPIB and Beclo, respectively, which were also significantly different.

Comparison of Beclo and two metabolites for induction of FACO and LH in cultured rat hepatocytes. M2 and M3 each induced FACO in a concentration-dependent manner in the same concentration range as Beclo when incubated with cultured hepatocytes for 72 hr (Fig. 3). Control activity after 72 hr incubation was 1.50 nmol $H_2O_2 \cdot min^{-1} \cdot$(mg protein). Maximal increases over control for Beclo, M2 and M3

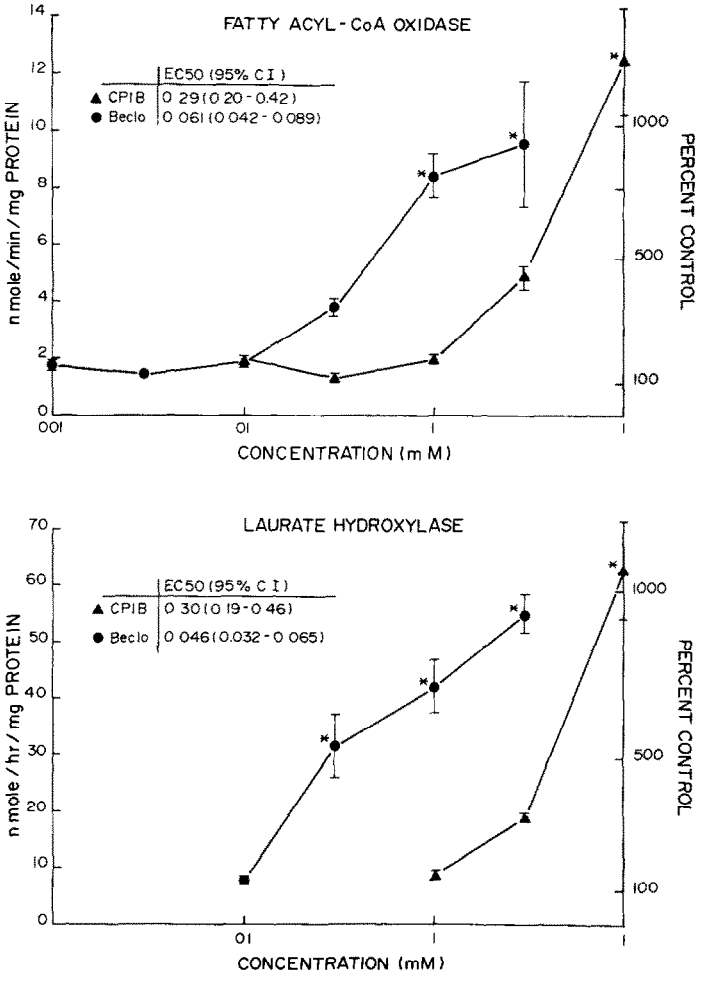

Fig. 2. Concentration–response curves for the induction of FACO (top) and LH (bottom) by CPIB (0.01 to 1 mM) and Beclo (0.001 to 0.3 mM). Hepatocytes were incubated for 72 hr in the presence or absence of drug. Results are expressed as absolute activities per mg cellular protein and as a percent of control. Each point represents the mean ± SE of determinations from 4 to 37 dishes of cells. Asterisks indicate significant induction relative to control. The EC_{50} values and 95% confidence intervals were determined by linear regression analysis using the following data points: Beclo (0.01 to 0.3 mM); CPIB (0.1 to 1 mM). Non-overlap of the 95% confidence intervals indicates a significant difference in potencies.

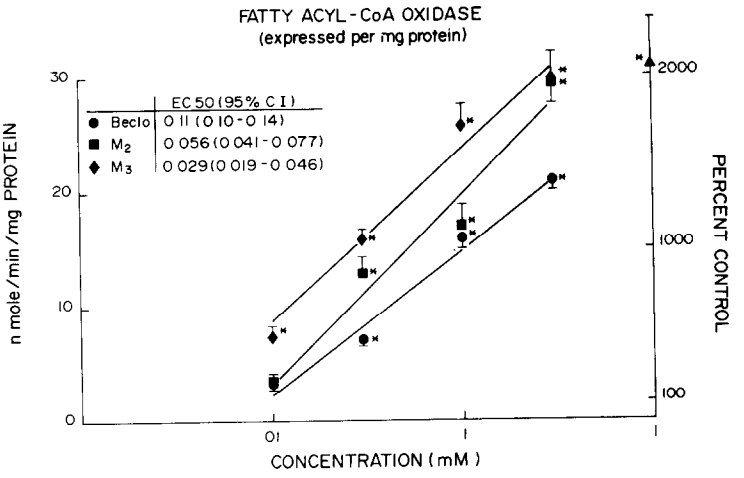

Fig. 3. Regression lines for the induction of FACO by Beclo, M2 and M3 (0.01 to 0.3 mM). Hepatocytes were incubated for 72 hr in the presence or absence of drug. Results are expressed as absolute activities per mg cellular protein and as a percent of control. Each point represents the mean ± SE of determinations from 4 to 8 dishes of cells. The closed triangle (▲) represents the effect of 1 mM CPIB. Asterisks indicate significant induction relative to control. Non-overlap of the EC_{50} 95% confidence interval of Beclo with those of M2 and M3 indicates significant differences in potencies. Overlap of the confidence intervals of M2 and M3 indicates no significant difference in potencies.

were 14- to 20-fold. The EC_{50} (95% C.I.) values (mM) determined by linear regression analysis (regression lines shown in Fig. 3) were 0.11 (0.10–0.14), 0.056 (0.041–0.077) and 0.029 (0.019–0.046) for Beclo, M2 and M3 respectively. Similar EC_{50} values and somewhat higher maximal increases over control (17- to 29-fold) were obtained when the data were expressed per mg DNA (data not shown). By overlap or non-overlap of the 95% confidence intervals, the order of potencies for the induction of FACO was M2 = M3 > Beclo.

M2 and M3 also induced LH in the same concentration range as Beclo when incubated with cells for 72 hr (Fig. 4). Control activity after 72 hr of incubation was 6.80 $nmol \cdot hr^{-1} \cdot (mg\ protein)^{-1}$. Maximal increases over control were 8- to 12-fold. In these experiments, the concentration–response curves for all drugs reached near-maximal responses at 0.03 mM. Since only two points were found on the linear portions of the concentration–response curves, linear regression analyses of these data could not be performed. Therefore, EC_{50} values were estimated

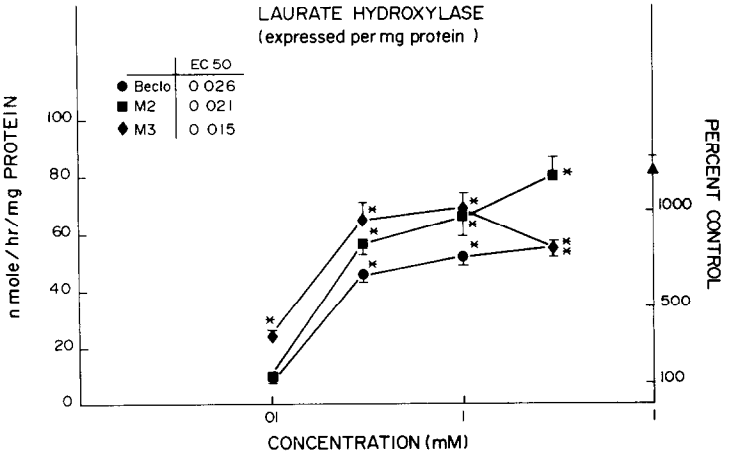

Fig. 4. Concentration–response curves for the induction of LH by Beclo, M2 and M3 (0.01 to 0.3 mM). Hepatocytes were incubated for 72 hr in the presence or absence of drug. Results are expressed as absolute activities per mg cellular protein and as a percent of control. Each point represents the mean ± SE of determinations from 4 to 8 dishes of cells. The closed triangle (▲) represents the effect of 1 mM CPIB. Asterisks indicate significant induction relative to control. The EC_{50} values were estimated from the plots.

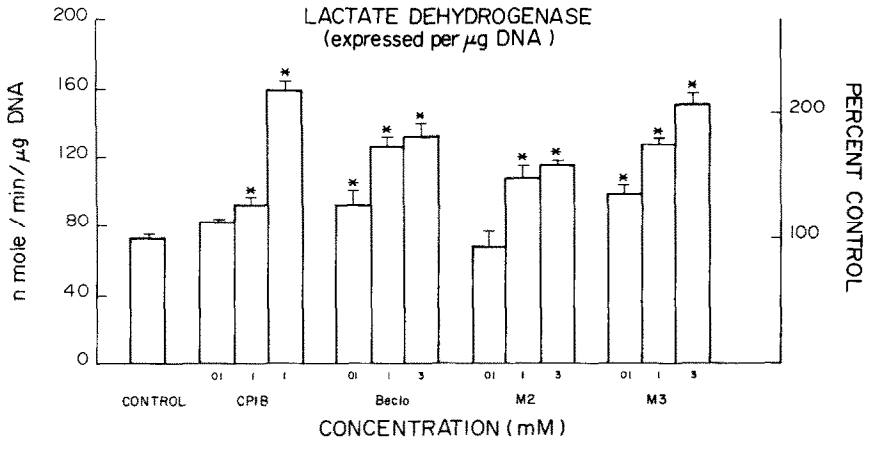

Fig. 5. Concentration–response histograms for the induction of cellular LDH by CPIB (0.01 to 1 mM), Beclo (0.01 to 0.3 mM), M2 (0.01 to 0.3 mM) and M3 (0.01 to 0.3 mM). Hepatocytes were incubated for 72 hr in the presence or absence of drug. Results are expressed as absolute activities per μg DNA and as a percent of control. Each point represents the mean ± SE of determinations from 3 to 8 dishes of cells. Asterisks indicate significant induction relative to control.

from the plots and gave values (mM) of 0.026, 0.021 and 0.015 for Beclo, M2 and M3 respectively. As for FACO, similar EC_{50} values and higher maximal increases (10- to 15-fold) were found when the data were expressed per mg DNA. Although no statistical analyses were done on these EC_{50} values, they followed the same trend as seen for FACO with M2 and M3 being more potent than Beclo.

Effects of drugs on protein and DNA content and lactate dehydrogenase (LDH) activity in cultured rat hepatocytes. CPIB, Beclo, M2 and M3 all caused significant concentration-dependent increases (22–53% at the highest concentrations tested) in the amount of total cellular protein per culture dish. Total cellular DNA per dish, however, was not affected significantly by any of the test compounds in our cell culture system (data not shown).

Each of these drugs produced concentration-dependent increases in cellular LDH activities after 72 hr of incubation with cultured hepatocytes (Fig. 5). The drug-induced increases were more evident when the data were expressed per μg DNA since DNA did not increase in concert with LDH activity as did cellular protein. Statistically significant increases relative to control were seen for each compound with maximal increases of 1.6- to 2.2-fold.

DISCUSSION

We have shown that (1) Beclo induced FACO and LH more potently than did CPIB in primary rat hepatocyte culture and (2) two phase I metabolites of Belco, M2 and M3, were more potent than the parent compound in their inductive effects. The abilities of Belco, M2 and M3 to function as peroxisome proliferators are not surprising considering their structural similarities to other potent peroxisome proliferating agents such as fenofibrate. In fact, M3 differs from fenobibrate only in the replacement of a side-chain methyl group with an ethyl group whereas an analogous difference exists for M2 and LF 2151, a reduced metabolite of fenofibrate which has been shown to induce carnitine acetyltransferase activity (another enzyme which is induced by peroxisome proliferators) in hamster liver [21].

Our *in vitro* data for enzyme induction by Beclo and CPIB agree reasonably well with *in vivo* hepatomegalic and lipid-lowering data for clofibrate and beclobrate [9]. Potency differences between Beclo and CPIB *in vitro* (4.8- to 6.5-fold) and between beclobrate and clofibrate *in vivo* (11- to 36-fold) may be attributable to such factors as (1) rat strain differences in the *in vitro* (Sprague–Dawley) and *in vivo* (Wistar) studies; (2) pharmacokinetic considerations for the drugs *in vivo* which are eliminated *in vitro*; and (3) a possible lack of association between enzyme induction and lipid-lowering activities [22–27].

While the potency differences between Beclo and its two methylene bridge-oxidized metabolites suggest that the presence or absence of oxygen at this position can influence peroxisome proliferating activity, definite quantitative conclusions regarding the relative inductive potencies of these compounds cannot be made from these studies. In contrast to the situation in humans, Beclo undergoes extensive metabolism in rats. Roth *et al.* [11] showed that only 17% of the radioactivity from a dose of [^{14}C]beclobrate is found in rat urine as Beclo, and M2 alone accounts for 17% of the radioactive products, Although the metabolism of Beclo has not been examined in rat hepatocyte cell culture, it is possible that at least a fraction of the inducing activity of Beclo in culture is actually mediated through its metabolites. Furthermore, Pourbaix *et al.* [21] have provided evidence that the reduction of fenofibrate to LF 2151 is a reversible process. It is therefore possible that the structurally similar M2 and M3 may themselves undergo interconversion. Thus, the overall enzyme-

inducing activity seen upon incubation of the cell cultures with Beclo, M2 and M3 may be influenced by one or both of the other species. Extrapolating to the *in vivo* situation, it seems possible that at least a portion of the lipid-lowering and/or hepatomegalic effects of beclobrate may be contributed by the oxidized metabolites of Beclo.

Our results regarding drug effects on cellular protein and DNA in culture are essentially in agreement with those of Gray *et al.* [28] who showed a much smaller increase in DNA than in protein using CPIB and BR-931. We report here that cellular LDH activity is increased in rat hepatocyte culture approximately 2-fold by CPIB, Beclo and its metabolites. Whether this effect can be seen *in vivo* remains to be determined as Waechter *et al.* [29] reported no induction of LDH by nafenopin in mice.

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