Antidiabetic Agents: A New Class of Reversible Carnitine Palmitoyltransferase I Inhibitors

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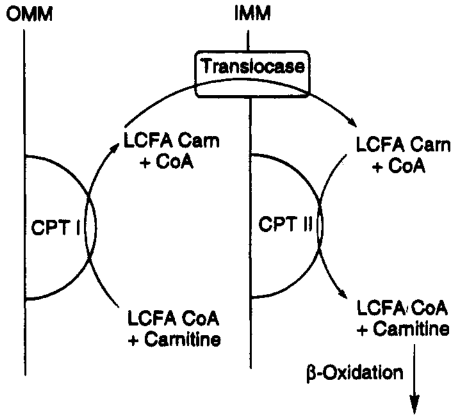
Inhibition of carnitine palmitoyltransferase I (CPT I) has been recognized as a mechanistic approach of potential clinical value in the treatment of non-insulin-dependent diabetes mellitus (NIDDM). This is because CPT I is considered the rate-limiting step in long-chain fatty acid oxidation[1,2] (Scheme 1), and in the NIDDM patient, particularly those with fasting blood glucose levels in excess of 200 mg/dL, hyperglycemia is primarily due to excess fatty acid oxidation driven hepatic glucose production (HGP).[3,4] These patients have high levels of plasma free fatty acids, which undergo hepatic oxidation to provide sufficient acetylcoenzyme A, ATP, and NADH for gluconeogenesis to proceed at maximal rates and thus for HGP to be excessive.[5] Inhibition of CPT I would inhibit fatty acid oxidation, decrease gluconeogenesis and HGP, and thereby decrease glycemia.

To date, two irreversible CPT I inhibitors have entered clinical trials, but both were subsequently dropped from development. These inhibitors are the glycidic acids tetradecylglycidic acid (TDGA)[6] and ethyl 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate (Etomoxir).[7] Etomoxir has been the most widely studied CPT I inhibitor and has been shown to be antihyperglycemic in clinical trials.[8−10] While the reasons for discontinuation of development of these agents have not been disclosed, it is likely that they were not further developed because they were shown to cause myocardial hypertrophy in animals. This toxicity results from these agents inhibiting fatty acid oxidation in heart muscle at the level of CPT I.[11]

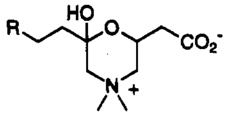
We hypothesized that a reversible, competitive inhibitor, selective for the liver versus heart isoform of CPT I,[12] *in vivo*, could prove effective at inhibiting fatty acid oxidation without inducing myocardial hypertrophy. At the time, aminocarnitine and (acylamino)carnitines[13,14] were known to be CPT inhibitors, presumably via a reversible mechanism, but limited information on these agents was available.

In order to rationally design reversible, competitive inhibitors of CPT I, transition state analog theory[15] was applied to the problem. While the details of the mechanism by which CPT I catalyzes the acyl transfer are unknown, kinetic studies with the highly homologous enzyme carnitine acetyltransferase (CAT) indicated a random bi-bi mechanism in which a covalent acyl enzyme intermediate could not be ruled out.[16] Assuming a similar mechanism for CPT I, we proposed the potential transition states depicted in Figure 1. These transition states were approximated by elimination of the membrane impermeable CoA and enzyme moieties and replacement of the tetrahedral ester oxyanion by a tetrahedral phosphonate anion to yield phosphonate **1** (Figure 1) as the parent of a new series of CPT I inhibitors. Subsequent to the initiation of our work, the CPT I inhibitor hemipalmitoylcarnitinium (HPC, **8**), also designed as a transition state analog, was reported by Gandour et al.[17] We report here some of our findings on a new series of CPT I inhibitors based on phosphonate **1**.

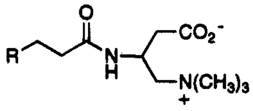
Scheme 1[a]



[a] Schematic of the carnitine palmitoyltransferase (CPT) system: CPT I, carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; CoA, coenzyme A; LCFA CoA, long chain fatty acid coenzyme A ester; LCFA Carn, long chain fatty acid carnitine ester; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.



8 (HPC) R = $C_{13}H_{27}$



9 (NPAC) R = $C_{13}H_{27}$

Phosphonates **1** and **2** and phosphonamidate **3** were prepared by coupling benzyl esters of the tetrafluoroborate or tetraphenylborate salts of carnitine and aminocarnitine to a phosphinic acid as shown in Scheme 2. The carnitine derivatives were prepared by esterification of the chloride salts of either carnitine or aminocarnitine with benzyl alcohol and HCl gas and then ion exchange with either $AgBF_4$ or $NaBPh_4$. The borate counterions are used to enhance the solubility of the carnitine derivatives in organic solvents. The phosphinate **4** was synthesized as shown in Scheme 3, and the phosphates **5** and **6** and the phosphoramidate **7** were prepared as shown in Scheme 4. In the case of **5**, **6**, and **7**, the tetrafluoroborate salts of the free carnitine acids could be used in the syntheses as the coupling reactions could be carried out in acetonitrile, a solvent in which the reaction components were readily soluble.

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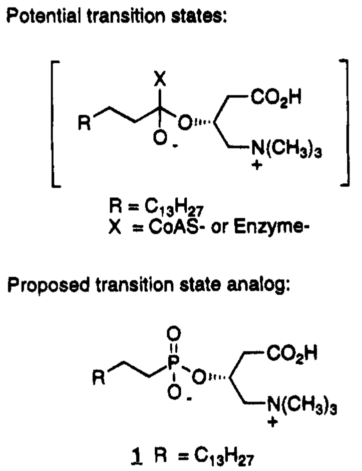
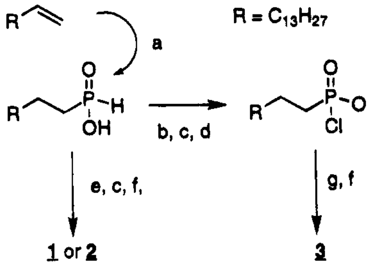

Potential transition states:
X
CO2H
R
O
O-
N(CH3)3
+
R = C13H27
X = CoAS- or Enzyme-
Proposed transition state analog:
1 R = C13H27

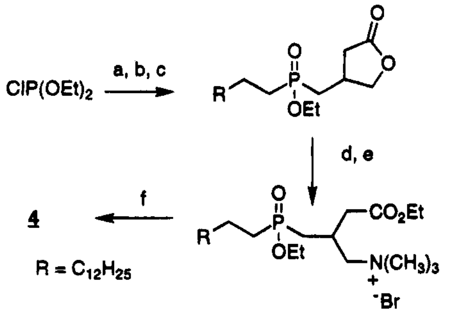
Figure 1. Potential carnitine palmitoyltransferase I enzymatic reaction transition states and proposed transition state analog 1.

Scheme 2[a]



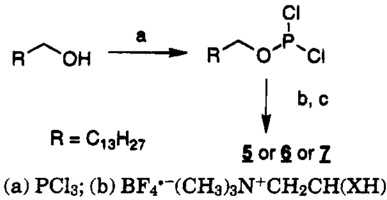
[a] Reagents: (a) NaH_2PO_2, AIBN, H_2SO_4, EtOH; (b) BnOH, DCC, DMAP, THF; (c) $NaIO_4$; (d) oxalyl chloride; (e) $BF_4^{\bullet-}$ $(CH_3)_3N^+CH_2CH(OH)CH_2CO_2Bn$, DCC, THF; (f) H_2, Pd/C; (g) $BPh_4^{\bullet-}$ $(CH_3)_3N^+CH_2CH(NH_2)CH_2CO_2Bn$, pyridine.

Scheme 3[a]



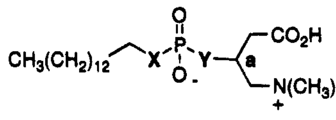
[a] Reagents: (a) $C_{14}H_{29}MgCl$; (b) 4-(bromomethyl)-2(5*H*)-furanone; (c) H_2, Pd/C; (d) HBr, EtOH; (e) NMe_3, THF; (f) NaOH, MeOH/H_2O.

Scheme 4[a]



[a] Reagents: (a) PCl_3; (b) $BF_4^{\bullet-}(CH_3)_3N^+CH_2CH(XH)CH_2CO_2H$, collidine, CH_3CN, where X = O for **5** and **6** and NH for **7**; (c) $NaIO_4$, H_2O.

Table 1. Inhibition of Fatty Acid Oxidation *in Vitro*



Compound	X	Y	a	IC50 (μM) homog[b]	IC50 (μM) hepat[b]
1	CH_2	O	*R*	36 ± 2.8	3.0 ± 0.64
2	CH_2	O	*S*	(>100)	(>300)
3	CH_2	NH	*R,S*	(>100)	(>300)
4		CH_2	*R,S*	(>300)	(>300)
5	O	O	*R*	28 ± 3.2	3.4 ± 0.50
6	O	O	*S*	(>300)	220 ± 82
7	O	NH	*R*	24 ± 3.0	46 ± 7.4
NPAC (**9**)				1.3 ± 0.30	1.2 ± 0.58

[a] Absolute stereochemistry at this center. [b] IC_{50} ± SE for inhibition of production of ketones in rat liver homogenates (homog) and isolated rat hepatocytes from 18 h fasted rats (hepat); data in parentheses indicates highest dose tested with no reasonable dose response being detected.

In vitro inhibition of fatty acid oxidation was measured by the reduction in production of ketones in rat liver homogenates and hepatocytes isolated from 18 h fasted rats.[18] As can be seen from Table 1, the parent phosphonate **1** was indeed active as an inhibitor of fatty acid oxidation in both the homogenate assay as well as the cellular assay. With the finding that **1** crosses cell membranes and inhibits fatty acid oxidation, further exploration appeared justified. The success achieved by Bartlett and colleagues[19] in designing thermolysin phosphinate and phosphonamidate inhibitors that were more potent than their respective phosphonate congeners, combined with the activity of the acylaminocarnitine *N*-palmitoyl aminocarnitine, (NPAC, **9**),[13] inspired us to examine atomic substitutions adjacent to the phosphorus atom of **1**.

It can be immediately seen from Table 1 that, in the cellular hepatocyte assay, the phosphonate **1** and the phosphate **5** are the most active whereas in the liver homogenate assay the order is slightly different, probably due to differing abilities of the compounds to cross cell membranes. Clearly, the phosphonamidate **3** and phosphinate **4**[20] are less potent than the phosphonate **1**, phosphoramidate **7**, and phosphate **5** in both assay systems. This is in direct contrast to the thermolysin results wherein phosphinate and phosphonamidate analogs were equipotent and these were more potent than a phosphonate analog due to desolvation and H-bonding effects, respectively.[19] Such effects are not evident in this system.

The possibility that these phospholipid compounds could be active due to their detergent-like physicochemical properties was of concern. However, as seen in Table 1, **2** and **6**, the *S* enantiomers, were much weaker than the natural and highly potent *R* enantiomers **1** and **5**. This militates in favor of a stereoselective binding as opposed to a nonspecific detergent effect.

In order to ascertain the utility of this series *in vivo*, compounds **1** and **5** were orally administered by gavage to 18 h fasted normal rats. In the rat, fasting results in a marked increase in hepatic oxidation of fatty acids to β-hydroxybutyrate and acetoacetate, with oxidation to carbon dioxide being of little quantitative importance.[21] This model, via measurement of serum β-hydroxybutyrate (β-HBA) levels, is therefore a valid *in vivo* model for the evaluation of inhibitors of fatty acid oxidation. In this model, compounds **1** and **5** reduced serum β-HBA levels at 3 h postdose with ED_{50}s of 24.5 and 4.2 mg/kg, respectively, with maximal suppression of these levels to about 20% of control. Thus, compounds **1** and **5** are orally active, efficacious inhibitors of fatty acid oxidation *in vivo*, with **5** being the more active analog.

Table 2. Effect of Phosphate **5** on Blood Glucose Levels in NIDDM Rats after 11 Days of Treatment[a]

dose (mg/kg/d)	glucose (mg/dL)	dose (mg/kg/d)	glucose (mg/dL)
0	197 ± 6	43.8	142* ± 16
21.9	164* ± 12	65.7	88** ± 7
32.9	179 ± 14	87.5	93** ± 10

[a] Blood samples were taken 6 h post-dose in a 6 h fasted state. (*$p < 0.05$; **$p < 0.01$, compared to control).

To assess **5** as an orally active hypoglycemic agent in an NIDDM model, phosphate **5** was orally administered by gavage once a day for 11 days to fat-fed low-dose streptozotocin-treated rats.[22,23] The data, shown in Table 2, indicate that at the higher doses **5** is capable of lowering blood glucose levels to near-normal values in this diabetic rat model.

The mechanism of action of **5** in the liver was examined *in vitro*, and the following results implicate inhibition of CPT I: (1) CPT I kinetic data obtained in isolated mitochondria are fully compatible with **5** acting as a reversible, competitive inhibitor of CPT I with respect to palmitoyl CoA ($K_i = 3.6 \pm 0.3\ \mu M$). (2) While phosphate **5** inhibits oxidation of palmitoyl CoA which requires CPT I for oxidation, it does not inhibit the oxidation of palmitoylcarnitine which bypasses CPT I. (3) Inhibition of fatty acid oxidation in rat hepatocytes by **5** is overcome by treatment of the cells with octanoic acid, a fatty acid oxidation substrate that enters the inner mitochondrial matrix directly, thereby bypassing CPT I. These data[24] indicate that **5** functions by inhibiting fatty acid oxidation in the liver at the level of CPT I.

The potential for phosphate **5** to induce myocardial hypertrophy *in vivo* was directly addressed by oral administration to normal rats at doses up to 100 mg/kg/day (~25-fold the ED_{50} for inhibition of hepatic ketogenesis in the 18 h fasted normal rat) for 26 weeks with monitoring of cardiac size and function by magnetic resonance imaging. Etomoxir was used as a positive control at 12.5 mg/kg/day orally. Over the course of the experiment, **5** had no effect on cardiac size or function whereas Etomoxir increased cardiac left ventricular mass by 10–15%.[25]

In summary, by employing transition state analog theory we have designed a new series of orally active, reversible, competitive inhibitors of CPT I. We have overcome the limitations of previous CPT I inhibitors by demonstrating that this new series is not prone to cause myocardial hypertrophy and hence is liver versus heart selective *in vivo*. Most importantly, these agents show promise for the treatment of NIDDM.

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