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PERSPECTIVE

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Carnitine Palmitoyltransferase (CPT) Modulators: A Medicinal Chemistry Perspective on 35 Years of Research

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INTRODUCTION

The metabolism of fatty acids, including their absorption, storage, mobilization, synthesis, and catabolism, has been the origin and target of innumerable drugs and pharmacological tools and the focus of countless research programs. The oxidation of fatty acids (FAO) is one of the most important cellular energy sources, and a pharmacological control on this process could have interest for a variety of therapeutic applications. In diabetes, the reduced sensitivity to insulin causes excessive release of fatty acids from the adipose tissue and increases their oxidation rate. This compounds the defects in tissue glucose uptake by promoting underutilization of glucose in the cells and overproduction of glucose by the liver, particularly due to excessive gluconeogenesis.[1] In the failing, overloaded heart, generation of energy by FAO poses heavy demands on the diminishing oxygen supply and promotes accumulation of potential toxic metabolites.[2] FAO inhibition in cancer cells that are heavily dependent on lipids as energy source has the potential to shut down their growth.[3] In dislipidemic and/or obese patients, on the other hand, increasing the FAO rate could curb the levels of circulating lipids and reduce tissue lipid storage, impacting favorably on body weight and the development of insulin resistance.[4] Finally, elements of the FAO machinery in the brain contribute to central control of energy homeostasis and feeding behavior.[5]

Most of the oxidation of long chain fatty acids (LCFAs) to acetyl-CoA occurs in the mitochondrial matrix. LCFAs are first converted to their CoA esters by the ATP dependent acyl-CoA synthases in the outer mitochondrial membrane, the cytosol, and the endoplasmic reticulum; however, the mitochondrial membrane is not permeable to long chain acyl-CoA (over ~C12). The mechanism by which LCFA access the mitochondrial matrix was elucidated by the pioneering studies of Fritz and Yue[6] and McGarry and Foster[7,8] and is illustrated in Figure 1. Acyl-CoAs are converted to acylcarnitine derivatives by the enzyme carnitine palmitoyltransferase 1 (CPT1) on the cytosolic face of the external mitochondrial membrane. Acylcarnitines are substrates for the shuttle-transporter carnitine acylcarnitine translocase (CACT), which mediates the transit of acylcarnitines from the cytosol to the matrix and the transport of free carnitine in the opposite direction.[9] Once inside the mitochondrial membranes, acylcarnitines are reconverted to acyl-CoA by the enzyme carnitine palmitoyltransferase 2 (CPT2) and can thus enter the FAO cycle.

McGarry and Foster established in 1980, on the basis of careful experimental data, that CPT1 is the controlling element of FAO rate and ketogenesis. The term "rate-controlling" is more appropriate than "rate-limiting", which has been used by some authors. Indeed, CPT1 activity is regulated by a considerable number of signals, which deliver feedback on the energy requirements of the organism and can vary over an extremely wide range. The variable basal activity of CPT1 and the difficulties in obtaining the isolated enzyme in active form have made it difficult to dissect the contribution of the various elements of the CPT system to FA transport and oxidation rate. FAO can also occur in microsomes and peroxisomes, which process LCFA down to the C8 length, after which they can enter the mitochondria via passive transport. Microsomes and peroxisomes possess their own CPTs, enzymes that are not well characterized but possibly are very similar if not identical to the mitochondrial CPT enzymes.[10] Peroxisomal oxidation can be induced in situations where the mitochondrial oxidation does not have sufficient capacity (for example, by high fat diet), and it has been demonstrated that the peroxisomal CPT is subject to similar controlling elements as the mitochondrial CPT system.[11] The contribution of peroxisomal CPT activity to observed FAO rates in cellular systems upon CPT inhibition is an incognita.

The lack of molecular modulators with well-defined activity and selectivity has been an added hurdle. Since the late 1970s, a few small molecules affecting the CPT enzymes, particularly inhibitors, have been identified. The oxirane carboxylic acids described by Tutwiler, Wolf, Sherratt, and Eistatter (BGLCF GmbH and McNeil) and the aminocarnitine derivatives described by Giannessi (Sigma Tau), Gandour, Anderson, and Griffith have been the most prominent and well characterized examples. These compounds have been used in an impressive number of in vitro and in vivo assays (including human clinical studies), assessing in particular potential effects in diabetes and cardiac failure. Nevertheless, or perhaps for this reason, a quantitative, consistent picture of relative and absolute activity, in vivo potency, selectivity, safety, and therapeutic potential of the various molecular entities is difficult to draft. In view of the complexity of the system, its sensitivity to a great number of factors, and the number of different setups that have been used to investigate its behavior, this is perhaps not surprising. It is important to stress at this stage that *all compounds for which data are reported in the literature are nonselective inhibitors and affect more than one isoform of CPT* (besides known or unknown off-target effects).

Interest in this target, as documented by scientific literature, has been slowly but steadily growing in the past decades (Figure 2). A few reviews have been compiled that cover CPT inhibitors.[12] This review will attempt to appraise the wide and tangled field of CPT modulators from the perspective of medicinal chemistry, using the chemotypes of CPT-interacting agents that have been described in the literature as a guiding beacon. Given the difficulty in obtaining quantitative comparable

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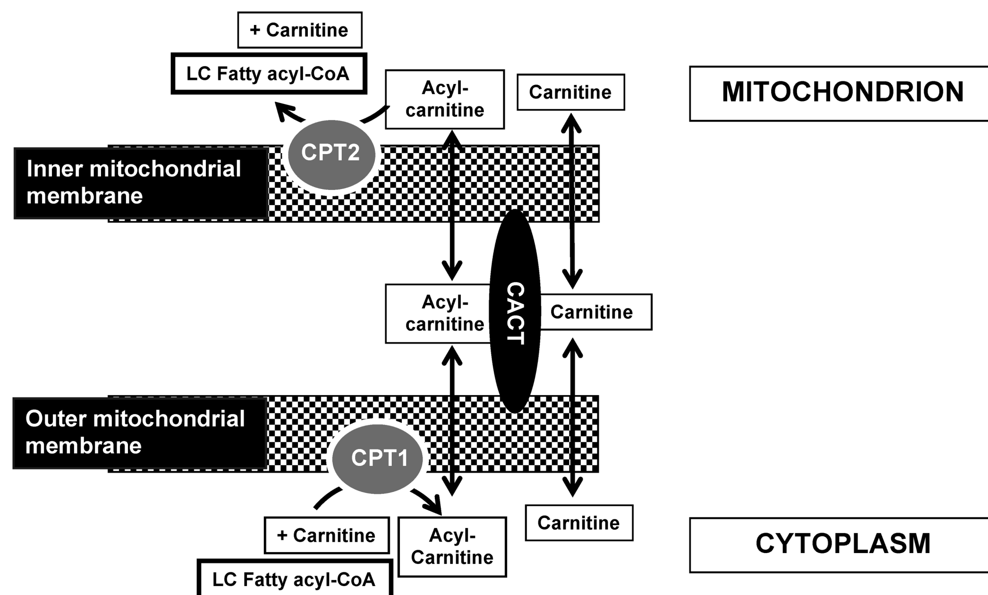

Figure 1. Schematic representation of the roles of CPT1, CPT2, and carnitine−acylcarnitine translocase (CACT) in shuttling long chain acyl-CoA derivatives through the mitochondrial membrane.

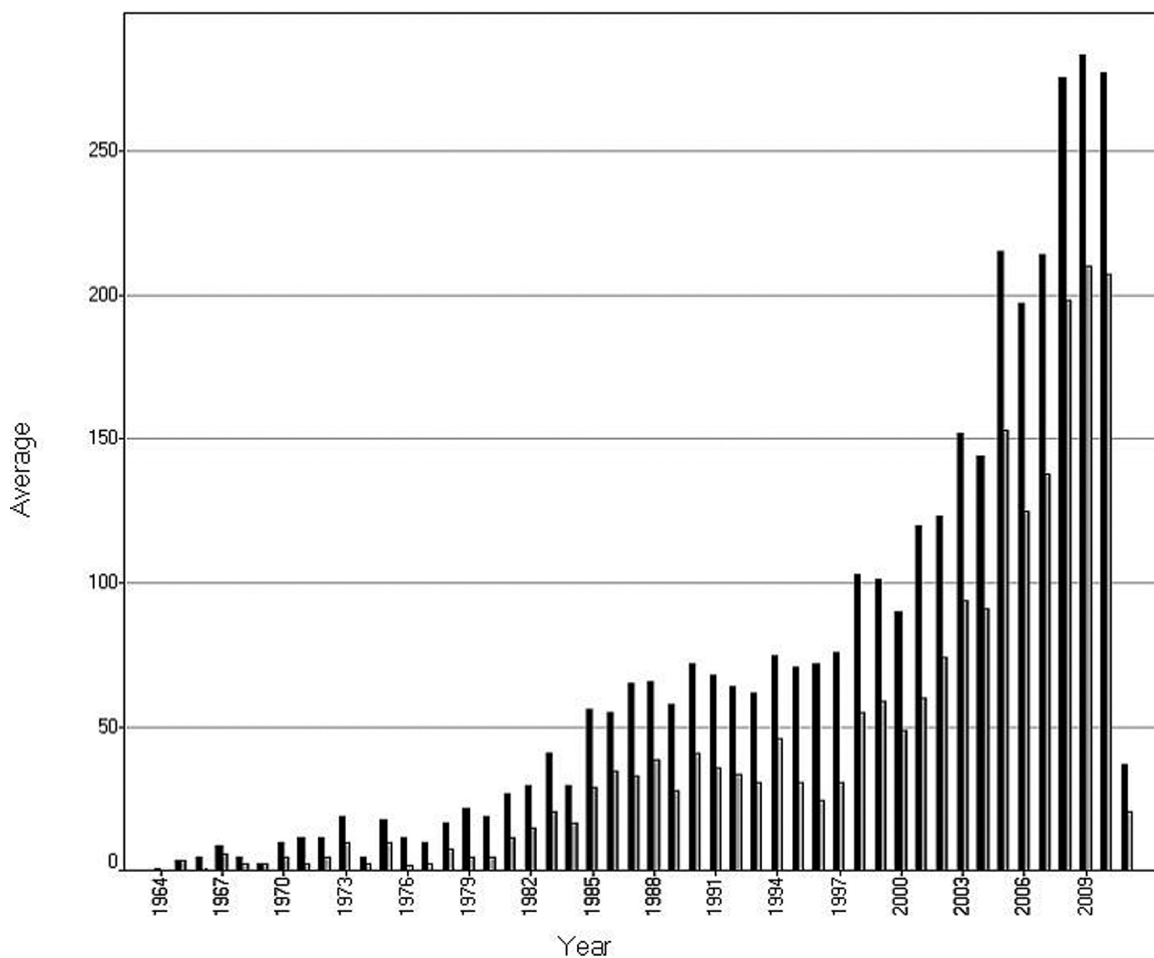

Figure 2. Histogram showing the recurrence of the term "carnitine palmitoyltransferase" (in black) in the title, abstract, or index of scientific literature and patents. The gray bars show the occurrence of the term "inhibitor" or "activator" in the data set. Source is the CAS database.

data for the activity and isoform specificity of these compounds, we believed that it would also be useful to include an evaluation of a few representative known pharmacological inhibitors of CPT in the same in vitro experimental setup (see Table 9).

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Table 1. Summary of All Known Proteins Belonging to the CPT family, Together with Their Synonyms and UniProtKB Database Numbers

abbreviated name	extended name[a]	human	rat	mouse	others
CPT1A (EC 2.3.1.21), CPT1A, CPT1-L	carnitine *O*-palmitoyltransferase 1, liver isoform	P50416	P32198	P97742	Q68Y62 (horse)
CPT1B (EC 2.3.1.21), CPT1B, CPT1-M	carnitine *O*-palmitoyltransferase 1, muscle isoform	Q92523	Q63704	Q924 × 2	Q58DK1 (bovine), Q8HY46 (pig)
CPT2 (EC 2.3.1.21)	carnitine *O*-palmitoyltransferase 2, mithocondrial	P23786	P18886	P52825	Q5U3U3 (zebrafish), Q60HG9 (cynomolgus monkey), Q2KJB7 (bovine)
CPT1C (EC 2.3.1.21), B-CPT1, CPT1-B	carnitine *O*-palmitoyltransferase 1, brain isoform	Q8TCG5		Q8BGD5	
CAT (EC 2.3.1.7), CrAT	carnitine *O*-acetyltransferase	P43155			
COT (EC 2.3.1.137)	peroxisomal carnitine *O*-octanoyltransferase	Q9UKG9			

[a] From the UniProtKB database: http://www.uniprot.org/.

Table 2. Tissue Distribution of the CPT Isoforms Determined by Northern Blot Analysis and, in Some Cases, by [^{13}C]-Etomoxir Labeling[13b]

tissue[a]	CPT1A	CPT1B	CPT1C	CPT2
liver	++++			+
skeletal muscle	(+)[b]	++++		+
heart	+	+++		+
kidney	++++	(+)[b]		+
pancreas	++++			+
lung	++++	(+)[b]		+
intestine	++++			+
brain	++++		+++	+
BAT	(+)[b]	++++		+
WAT	+	+++		+
testis	(+)[b]	++++		+
ovary	++++	(+)[b]		+
skin fibroblasts (human)	++++			+
leukocytes	++++			+

[a] All tissues are rat tissues, with the exception of skin fibroblasts. [b] The symbol "(+)" indicates that trace expression has been detected.

In this discussion, we will attempt to cast some light on why a target with such a long history and such a central role in energy metabolism has failed so far to deliver a drug, despite several worthy attempts, and whether its potential to be leveraged for therapeutic application still has to be fulfilled.

CPT ISOENZYMES: GENERAL AND STRUCTURAL ASPECTS[13]

The CPT family comprises four currently known isoforms, which are denominated CPT1A, CPT1B, CPT1C, and CPT2. A summary of all known proteins belonging to the CPT family and related enzymes is provided in Table 1, together with their filing numbers in the UniProtKB database.[14]

The isoforms differ in their intracellular location, their tissue distribution, and their sequence. The CPT1 isoforms are located on the cytosolic side of the external mitochondrial membrane and are anchored to the membrane via a transmembrane portion comprising two hydrophobic stretches of amino acids at the N-terminal side of the protein. The N-terminus itself (~47 residues) is also located on the cytosolic side, giving the protein a hairpin polytopic conformation. The only part of the protein exposed to the intermembrane space is predicted to be a 27-residue loop.[15] The CPT2 isoform is associated with the internal mitochondrial membrane and extends and operates into the intramitochondrial space.[16] These subcellular locations are perfectly in line with their role in catalyzing the direct and the reverse reaction converting acyl-CoA into acylcarnitine. CPT1A and CPT1B have different enzyme kinetics. CPT1A has a much higher affinity for carnitine (K_m = 30 μM for the rat enzyme) than CPT1B (K_m = 500 μM for the rat enzyme).[9]

The most relevant distinction between the family members from a medicinal chemistry perspective, however, is their tissue distribution. As shown in Table 2, CPT1B is strongly expressed in skeletal and heart muscle as well as in adipose tissue and testis but totally absent in all other organs involved in FAO. The striking aspect of CPT1A distribution, on the other hand, is its absence from skeletal muscle and adipose tissue. CPT1A is modestly expressed in heart muscle but does not seem to play a significant role in lipid oxidation in that organ in adult organisms.[17] In juvenile organisms, however, CPT1A is heavily involved in the control of heart lipid oxidation.[18] CPT1A is the only CPT1 isoform present in the liver. For this reason, CPT1A is often referred to in the literature as liver CPT1 or L-CPT1, while CPT1B is often called muscle CPT1 or M-CPT1. This descriptive nomenclature can be misleading, however, as it understates the role of CPT1A and B in other organs besides liver and muscle, a consideration that is particularly relevant, for example, for evaluating the safety of isoform-specific CPT inhibition. For this reason, the nomenclature CPT1A and CPT1B will be used in this review. CPT1C appears to be expressed exclusively in the brain, where it may act as a buffer of malonyl-CoA concentration rather than a catalytically active enzyme.[19,20] A unique CPT2 isoform, on the other hand, is ubiquitously expressed in all tissues.

Selectivity toward each of the CPT family members and tissue distribution are very central descriptors for any small molecule modulator of the CPT enzymes, impacting the macroscopic effect in healthy and diseased states and the efficacy and the safety of a putative therapeutic intervention. Human CPT1A and CPT1B share a 63% sequence homology (Table 3a), although the homology in the active site is notably higher (82%) and the sequence similarity is close to 80%. CPT2 on the other hand is shorter (lacking the membrane-anchoring domain, 658 residues vs 773 for hCPT1A)[13b] and more different, with only 36%

Table 3. Total/Active Site Homology

(a) Total/Active Site Homology of Members of the Human CPT Family, Carnitine Acetyl Transferase (CAT), and Carnitine Octanoyl Transferase (COT)[21,a]

	CPT1A	CPT1B	CPT1C	CPT2
CPT1B	63.1/81.8			
CPT1C	55.3/63.6	53.5/54.5		
CPT2	22.7/36.4	24.7/41.8	22.8/36.4	
CAT	22.5/38.2	23.1/40.8	22.1/36.4	23.0/49.1
COT	21.3/29.1	21.6/29.1	20.0/29.1	20.2/30.9

(b) Total/Active Site Homology of CPT1A across Human (h), Rat (r), and Mouse (m)

	hCPT1A	rCPT1A
rCPT1A	86.7/91.1	
mCPT1A	86.5/92.0	95/99

[a] Homology was calculated with MOE 2009.10 default settings.

homology in the active site. These data would point a medicinal chemist to expect that it should be relatively easy to gain selectivity between CPT2 and CPT1 inhibitors, while it might prove to be a daunting task for a competitive inhibitor to differentiate CPT1A from CPT1B. An alternative option is to achieve tissue selective distribution of an unspecific inhibitor via permeability and pharmacodynamic properties.

The homology between human, rat, and mouse enzymes, on the other hand, is rather high (see Table 3b), and species differences at the in vitro level are not likely to be significant (the rate of FAO in different species and the level of CPT activity control can be considerably different).[22] CPT1 is subject to multiple controlling factors. Expression level and basal activity of CPT1 depend on fasting state,[23] exercise,[24] type of diet,[25] exposure to cold, infections, metabolic disease, and enzyme inhibition.[9] Diabetes does not affect CPT1B activity[26] but increases CPT1A activity.[27] Malonyl-CoA, the first committed step of fatty acid synthesis, whose concentration is in turn highly regulated by multiple mechanisms, is a potent inhibitor of CPT1B (~0.03 μM IC_{50} for the rat enzyme) and a less potent inhibitor (by about 2 orders of magnitude) of CPT1A.[28,29] The sensitivity of CPT1A to allosteric inhibition by malonyl-CoA varies under different physiological conditions, amplifying the effect of the cellular malonyl-CoA concentration. In particular, declining malonyl-CoA concentrations reduce the sensitivity of the enzyme to allosteric inhibition (and vice versa). Mitochondrial outer membrane composition also affects sensitivity of CPT1A to malonyl-CoA. Studies regarding the binding of malonyl-CoA and other active-site directed inhibitors have shown that there are two separate binding sites for malonyl-CoA: a high-affinity binding site located on the cytoplasmic side of the protein and a second low-affinity site that corresponds to the catalytic site and where also CoA exerts a product-inhibition action.[30] It is postulated that one of these binding sites is a contact interaction between the N- and C-terminal segments of the protein, which explains the sensitivity to membrane fluidity as well as the loss of sensitivity to malonyl-CoA if the N-terminal portion of the protein is deleted (which also leads to loss of function) or by specific single point mutations in this region. The N-terminal domain is also responsible for the targeting of the enzyme to the outer mitochondrial membrane.[31] There is evidence that CPT activity is controlled by phosphorylation[32] and nitration,[33] although the hypothesis has been advanced that CPT is constitutively phosphorylated.[28]

Under these circumstances, it is not surprising that it is very difficult to obtain precise and consistent data on the absolute and relative potency of CPT1 inhibitors, as the results obtained are likely to be highly dependent on the origin and handling of the biological matrix and the precise assay conditions.

The brain specific isoform CPT1C binds malonyl-CoA with a K_d similar to that of CPT1A (~0.3 μM).[34] CPT2, on the other hand, is not subject to allosteric inhibition by malonyl-CoA, although it has been reported that its activity and expression levels vary in response to physiological status.[13b]

It has been proven difficult so far to solubilize CPT1 or a construct thereof without losing enzyme activity, which is certainly due to the importance of the correct morphology of membrane anchoring and of membrane status for activity. Consequently, no structural information on the CPT1 enzymes is available. The crystal structure of rat CPT2, on the other hand, has been deposited.[35] This allows some considerations on the binding site characteristics of the CPT enzymes, which are likely to be quite similar across the isoforms. As postulated by Ramsay et al.,[36] CPT2 possesses three binding sites for CoA, acyl, and carnitine (see Figure 3), which together form a Y-shaped tunnel at the interface of the C-terminal and N-terminal domains.[37] The acyl and the CoA tunnel open to the surface of the protein. The CPT inhibitor **19** (ST1326)[120] (see Scheme 2) has been cocrystallized with CPT2 and occupies the acyl and the carnitine sites, showing which residues are relevant for binding to carnitine. As shown in Figure 3, His372, which is conserved throughout the carnitine/choline acyltransferase family and is essential for catalytic activity of CPT2,[38] forms a hydrogen bond with the amino nitrogen (N11) of **19**, which substitutes the ester oxygen of the native ligand palmitoylcarnitine. Ser590 of the Ser-Thr-Ser motif conserved among carnitine acyltransferases makes a hydrogen bond to the carbamoyl oxygen (O13) of **19**, which would be an ester carbonyl in the substrate. Tyr486, Ser488, and Thr499 of the carboxy terminal domain, as well as a conserved water molecule, bind the carboxylic group of the carnitine-like head of **19**. Arg498 forms a strong hydrogen bond with the side chain of Asp376, and its guanidinium group interacts with the main chain carbonyl oxygen of Ser373 in the catalytic loop, thereby positioning the active site residues in an ideal position for catalysis. The positively charged tertiary amine is stabilized by cation−π interactions with the conserved Phe602. The hydrophobic tunnel that accommodates the acyl tail is lined by residues of β strands 1 and 16, which form an antiparallel β sheet at the domain interface.

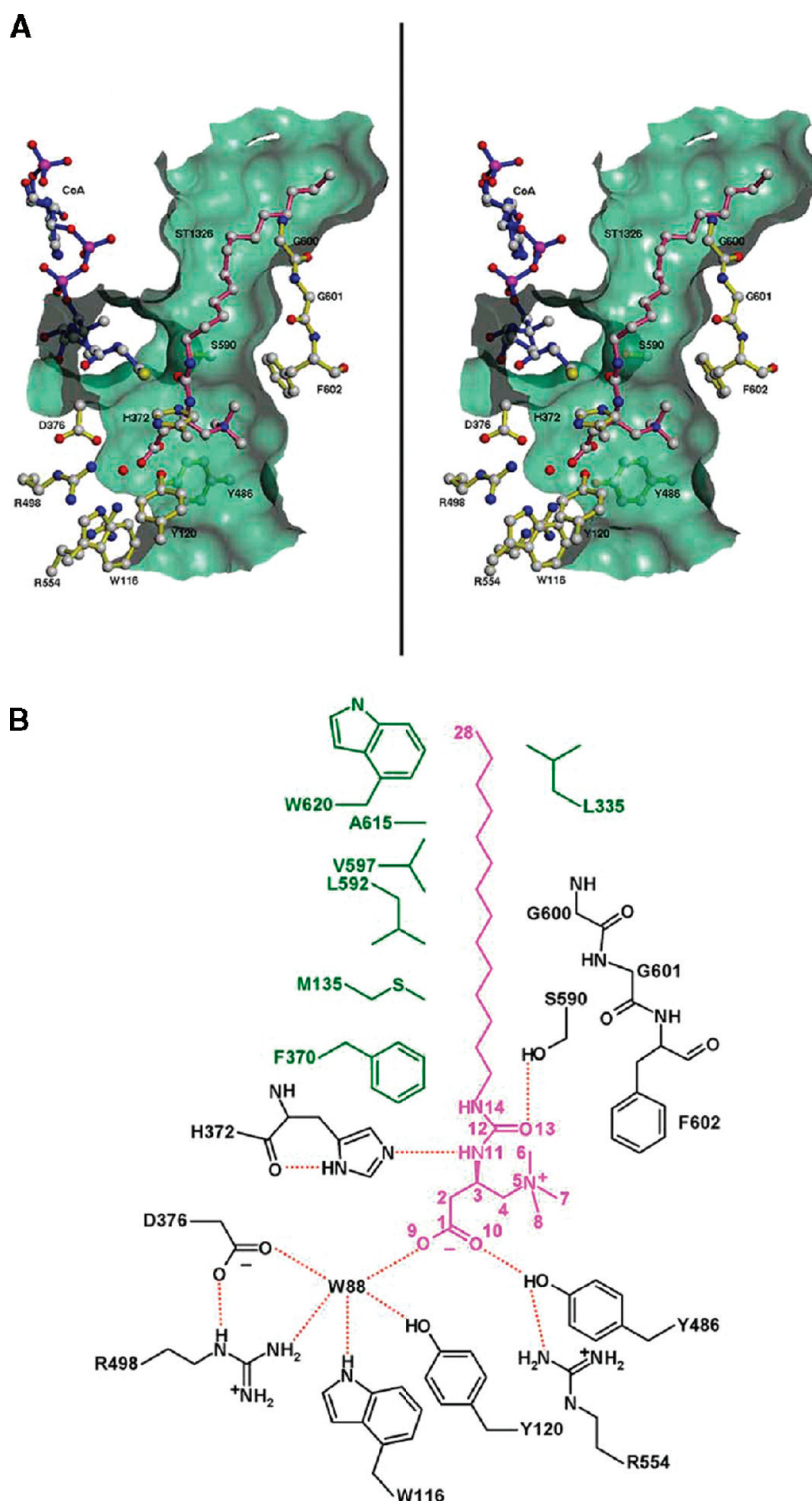

Figure 3. (A) Stereoview (generated with MOLOC[21]) of the tripartite active site tunnel with bound **19** viewed perpendicular to the domain interface. Key active site residues are depicted in yellow. The cocrystallized **19** is shown in pink. The CoA molecule (blue) was modeled based on the CoA coordinates from the CAT-CoA complex structure (PDB code 1t7q). The protein environment of the modeled CoA molecule was omitted for clarity. (B) Projection of **19** (pink) with atom numbering as used in the text. Key interactions of the hydrogen bond network (red dashed lines) and with hydrophobic residues (green) are depicted. Parts A and B are reproduced with permission from *Structure*.[35b] This article was published in *Structure*, **2006**, *14*, Rufer, A. C.; Thoma, R.; Benz, J.; Stihle, M.; Gsell, B.; De Roo, E.; Banner, D. W.; Mueller, F.; Chomienne, O.; Hennig, M. The crystal structure of carnitine palmitoyltransferase 2 and implications for diabetes treatment, 713−723. Copyright Elsevier 2006.

A homology model of CPT1A employing the structure of CAT as template has been reported.[39] The CPT2 crystal structure offers itself as closer analogue to be used for this exercise in the future.

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Mitochondrial preparations from yeast expression systems have been used in the past decade to obtain separated recombinant CPT isoforms that can be used to study the differential effect of small molecules on each enzyme of the CPT family. The yeasts (*Pichia pastoris* or *Saccharomyces cervisiae*) deliver enzymes that have properties similar to those derived from native sources and have the advantage that they do not have endogenous CPT activity.[40]

CPT INHIBITORS

Most of the compounds known to affect the CPT enzymes are inhibitors. The field of CPT inhibitors has been the victim of peculiar medicinal chemistry neglect. The target is very well-known for several decades, and the rationale for both inhibition and activation is given. It is a highly "druggable" target, and in the case of CPT2, even structural information has been available for a few years. Pharmacological intervention in the process of FAO may raise safety concerns but has a compelling rationale in a number of indications. Nonetheless, the medicinal chemist scanning the literature for CPT inhibitors will find only a handful of chemotypes, with the majority falling into two categories: oxirane carboxylic acids or acylcarnitine analogues (substrate analogues). One may be led to the conclusion that inhibition of CPT with small molecules is strewn with unfathomable difficulties. Quite the contrary is the case. Recently, the authors have filed several patents demonstrating multiple chemically diverse series of small molecule CPT inhibitors with various specificity, whose details will be published in due course (see Table 8 and references therein).

From the point of view of mechanism of action, most CPT inhibitors bind at the active site, while a few have been reported to bind at the malonyl-CoA allosteric site. Oxirane carboxylic acids are irreversible inhibitors, which need to be converted to CoA derivatives in physiological conditions before exerting their action. Inhibition by carnitine derivatives and other noncovalent active-site directed inhibitors is competitive in nature. Oxalic acid derivatives (compounds **51** and **52**) are the only reported noncompetitive CPT inhibitors.

Appraisal of quantitative literature data on CPT inhibitors is made difficult by a number of factors. In vitro, apparent IC_{50} values are strongly dependent on the specific experimental conditions, the type of assay used (radiometric vs photometric vs chromatographic), and the type of analyte chosen. Moreover, until recently, tissue preparations, whole cells, and mitochondrial preparation from rodents or other animal species were used to generate quantitative in vitro data, each giving a different type of readout. In vivo, the nutritional status and endocrine status of the animals and the species affect the range as well as the sensitivity and the actual value of the biomarkers used. Although the assay systems for investigating isoform specificity have been available for a few years, almost no such information has been reported in the literature.

The following paragraphs offer a critical overview of the main classes of CPT modulators reported in the literature. Given the lack of comparative information, we have also generated data for selected members of each structural class in the same set of in vitro assays, including isoform specificity (see Table 9).

Oxirane Carboxylic Acids[41]**.** Following reports on CPT inhibition by modified alkanoic acids like 2-bromopalmitoyl-CoA,[42] oxirane carboxylic acids were among the first inhibitors of carnitine-dependent FAO to be extensively profiled in the literature. Representative structures of this class of compounds are reported in Scheme 1. As the compounds have appeared repeatedly in the literature in the past 30 years, a certain confusion has originated on the use of generic names. In this and in following schemes, the most common names and codes used for each compound in the literature are reported: the one underlined will be used in the present document.

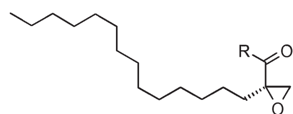
All oxirane carboxylic acids bear a stereogenic center at C_2. It has to be assumed, based on the reports available, that the most active isomer is consistently the (*R*)-isomer, as indicated in Scheme 1. Many of the experiments, however, have been performed with racemates, or the stereochemistry has not been specified. In the text and tables the stereochemical information relevant to the experiments discussed will be added before the compound number. Although they readily undergo nucleophilic addition to the protein (see below), these compounds, as many aliphatic oxiranes, are chemically stable.

Table 4 reports representative in vitro and ex vivo studies that have been performed with oxirane carboxylic acids. The results are reported in a summarized form, and the reader is encouraged to refer to the original literature for more details. Analogously, Table 5 reports an almost comprehensive overview of in vivo experiments, including clinical studies. As evident from the tables, these compounds have been characterized especially as inhibitors of gluconeogenesis and ketogenesis (see section on therapeutic potential) and in models of cardiac ischemia and diabetes.

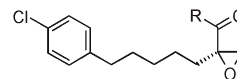
Oxirane carboxylic acids represent a very homogeneous structural class and constitute probably the most potent traditional CPT1 inhibitors. The tens of papers published on these compounds in the years 1980−2000 tend to repeat the same experiments multiple times in different settings and with only marginally different compounds, reconfirming the chemical, biological and physiological behavior of this type of CPT1 inhibitors and the general principles of pharmacological intervention on the CPT system.

In a pivotal 1978 paper, Tutwiler et al. from McNeil Laboratories detailed the effect of a surprisingly potent hypoglycemic compound, methyl 2-tetradecylglycidate (**1a**, McN-3716), in rats and dogs (see Table 5).[46] The compound was correctly identified as a fatty acid oxidation inhibitor, as it did not affect glucose levels in animals that were fed a carbohydrate rich diet, while it had impressive effects in fasted animals or animals on a low carbohydrate-high fat diet. In these studies, **1a** was shown to be much more potent than tolbutamide and phenformin in lowering fasting blood glucose. A year later, the first indications of **1a** being an irreversible, covalent inhibitor of CPT were reported, as well as some limited SAR demonstrating the specific inhibitory effect of **1a** over analogous inactive compounds (for example, the corresponding cyclopropane and the corresponding alcohol).[47,48] In these reports, it was shown that **1a** inhibits to a much greater extent the oxidation of long chain fatty acids, like oleate, stearate, and palmitate, than oxidation of laurate and other short chain fatty acids. Oxidation of palmitoylcarnitine was not inhibited. Inhibition of ketogenesis and gluconeogenesis by **1a** could not be reversed by addition of CoA or carnitine, excluding that depletion of these cofactors participates in the mode of action. Acyl-CoA synthase was inhibited by **1a** but only in millimolar concentrations. On the basis of these observations, the conclusion was reached that **1a** is an inhibitor of CPT1, a reasoning that was later confirmed by experiment.[49] The relationship between inhibition of FAO and inhibition of glucose production in hepatocytes from fasted animals was also established. The -SCoA derivative of the

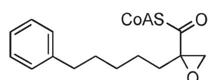
Scheme 1. Structures of Representative Oxirane Carboxylic Acids with the Most Common Use Names or Codes by Which They Appear in the Literature[a]



1a R = OMe: methyl palmoxirate, tetradecylglycidic acid methyl ester, Me-TDGA, McN-3716
1b R = OH: tetradecylglycidic acid, TDGA, palmoxirate, McN-3802
1c R = SCoA: TDGA-CoA



2a R = OEt: clomoxir, POCA-ethyl ester, B807-06, WY49422
2b R = OH or R = O⁻Na⁺, clomoxir, POCA, B807-27
2c R = SCoA: POCA-CoA



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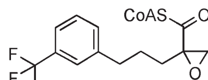

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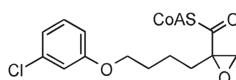

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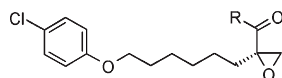
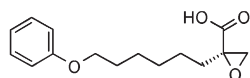
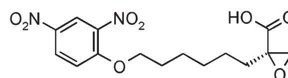


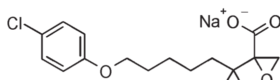
6a R = OEt: etomoxir ethyl ester, etomoxir, B807-54
6b R = OH: etomoxir, B807-33
6c R = SCoA: etomoxir-CoA



7: dechloroetomoxir, DET, B847-47



8: dinitrophenyl-etomoxir, DNP-Et



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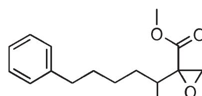

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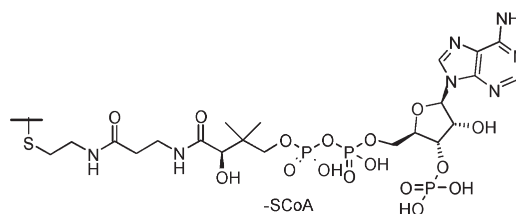

[a] Underlined is the common name used in the present manuscript.

(R)-isomer of **1a**, compound **1c**,[50] was later confirmed as the active form of **1a** in vivo and in cellular preparations containing the appropriate cofactors (Mg^{2+}, ATP, and CoA as well as either mitochondrial or microsomal acyl-CoA synthetase). The (R)-enantiomer is converted to its CoA ester 9 times faster than the (S)-enantiomer. It is not clear, therefore, whether the higher activity is due to intrinsic potency at CPT1 or to a faster conversion of the prodrug to the active species (although this proof is available for related compounds; see below). Proof of irreversible binding to the active site of CPT1 was obtained via accurate studies of enzyme kinetics and with the use of 3-^{14}C labeled **1c**.[51] A number of in vivo evaluations confirmed a strong hypoglycemic and hypoketotic effect in several animal species, including humans (see Table 5). Syntheses of enantiopure **1a**,**b** have been reported.[52] Some discussion of the SAR around compounds **1** was reported, although a more potent analogue was not identified.[53]

Very similar and almost equally potent inhibitors of the transport of FA across the mitochondrial membranes were identified by Eistetter and Wolf. The most prominent examples of such compounds are compounds **2** (POCA), in which the C14 side chain of **1** is substituted by a short C5 alkyl chain bearing a 4-chlorophenyl group.[54,55] The demonstration that compounds **2** inhibit both CPT1A and CPT1B via the intermediacy of the -SCoA derivative was delivered by Bartlett et al.[56,72] Compound **2c** (POCA-CoA) was also found to be an inhibitor of fatty acid synthase, although at much higher concentrations than those at which it inhibits FAO.[57] Interestingly, in isolated liver mitochondria from fed animals compounds **2** are more active than in liver mitochondria isolated from fasted animals.[58] This effect is also observed for malonyl-CoA,[59] for which sensitivity is lower in mitochondria isolated from animals in a more ketogenic state.[28] This analogy suggests that oxirane carboxylic acids and malonyl-CoA share binding site(s) and/or mode of action. For muscle mitochondria there is no difference between mitochondria extracted from fed or fasted animals. Although it is difficult to judge the relative potency of compounds measured by so many different methods and setups, it appears that a couple of more potent analogues of **2** were identified by Sherratt et al., for example, compound **5**.[58] While the C6 and C7 analogues of **2** were considerably less potent, insertion of an oxygen in the C7 chain led ultimately to the most well-known and described of all CPT1 inhibitors based on the oxirane carboxylic acid unit: compound **6a** (etomoxir ethyl ester).[60] This compound has grown to be one of the most widely used tool compounds for the study of mitochondrial β-oxidation and has drawn attention as a potential drug in a number of indications. As is the case for **1**, the (R)-enantiomers of compounds **6** are much more potent than the (S)-enantiomers both in vitro and in vivo. Compound **6c** (etomoxiryl-CoA), the active form of **6a** and **6b**, was shown to inhibit also other carnitine transferases, in particular the liver microsomal enzyme COT ($IC_{50} \approx 1.5\ \mu M$) and the peroxisomal carnitine acyltransferase(s) COT and CAT, with tissue specific K_i.[61] As reported for other oxirane carboxylic acids, inhibition potency depends on incubation time, tissue, and species, and it is difficult to quantify or compare, although it is likely to be in the low nanomolar range under appropriate assay conditions. Compound **6a** has a half-life of ~1.5 h and is metabolized via hydrolysis of the oxirane ring followed by degradation of the chain, leaving 4-chlorophenoxypropionate as the main metabolite.[62] The enzyme occupancy, however, must last considerably longer. Several enantioselective[63] and racemic[64] syntheses of

Table 4. Summary of in Vitro and ex Vivo Pharmacological Assays Performed with Oxirane Carboxylic Acids

compd	compd name	type of assay	biological matrix	assay conditions	efficacy	ref
rac-**1a**	Me-TDGA	subcellular	rat liver mitochondria	Formation of palmitoyl[^{3}H]carnitine from palmitoyl-CoA and [^{3}H]carnitine. Conditions as in ref 28 plus ATP and CoA in reaction media and preincubation time of 5 min	IC_{50} = 0.046 μM	51
		tissue and ex vivo	hemidiaphragms isolated from fasted pretreated rats (10−15 mg po)	[^{14}C]-Glucose and [^{14}C]-fructose (5 mM), [3-^{14}C]-BHB + FFA-albumin solution (0.3 μM FFA, 0.5 μCi)	Inhibition of $^{14}CO_2$ production from [1-^{14}]-palmitate or [^{14}C]-oleate($EC_{50} \approx 1$ μM). Acceleration of [U-^{14}C]-glucose oxidation (10 μM to 1 mM). Lag phase (20 min) eliminated by preincubation in tissue studies.	47
rac-**1b**	TDGA	subcellular	rat liver mitochondria	Mitochondria suspended in mixture containing 15 μM CoA and 5.9 mg/mL BSA. Preincubation with TDGA in ethanol, then 0.1 μmol of palmitoyl-CoA and 0.2 μmol of labeled carnitine added.	Inhibition of conversion of palmitoyl-CoA and [^{3}H]CH_3-L-carnitine into palmitoyl[^{3}H]-CH_3-L-carnitine. IC_{50} depends on preincubation time (∼0.25 μM at 2 min)	49
			SD rats liver mitochondria	Formation of palmitoyl[^{3}H]carnitine from palmitoyl-CoA and [^{3}H]carnitine. Conditions as in ref 28 plus ATP and CoA in reaction media and preincubation time of 5 min.	IC_{50} = 0.048 μM	51
		cellular	hepatocytes isolated from 48 h fasted rats	Substrate concentration: 1−2 mM	Inhibition of $^{14}CO_2$ production from [1-^{14}C]-palmitate, $EC_{50} \approx 1$ μM. No effect on oxidation of octanoate or palmitoylcarnitine. Inhibition of KB production from oleate, at concentration ≥ 0.1 μM. Strong reduction of long-chain acylcarnitine levels, marked depletion of acetyl-CoA.	48
			hepatocytes isolated from 48 h fasted rats	1% BSA and 0.7 mM oleate, 1−2 mM substrate	Inhibition of glucose formation from pyruvate, lactate, dihydroxyacetone, and fructose, EC_{50} = 1−10 μM, decrease of KB production. Strong decrease of lactate/pyruvate ratio. Inhibition of glucose formation from pyruvate by 10 μM **1** reversed by addition of 2 mM octanoate.	48
			hepatocytes isolated from fed rats	Substrate concentration: 2 mM for pyruvate, 0.7 mM for oleate	No effect on glucose production and KB production either from endogenous substrates or from pyruvate. Effect on gluconeogenesis and KB production from oleate.	48
		tissue	perfused rat hearts	1 mM oleate, 2% albumin. 35% perfusate oxygen content in hypoxia. Addition of TDGA 40 min before addition of oleate.	Inhibition of oleate oxidation, EC_{50} = 3−4 μM. No effect at low work-loads, inhibition of LVDP and aortic output at high work loads. In hypoxic hearts, reversal of deleterious effect of oleate addition (reduction of anoxic areas) (10 μM TDGA)	79
			perfused rat heart	Diabetic and nondiabetic rat hearts perfused with glucose (11 mM) and palmitate (0.1 mM)	In mild ischemic conditions (15 min) addition of TDGA to perfusate 10 min before addition of palmitate reduces lactate dehydrogenase (LDH) release and tissue accumulation of long-chain acylcarnitine in both type of hearts. Significant improvement in mechanical function with TDGA only in ischemic diabetic hearts.	81
			perfused guinea pig livers	Livers from 48 h fasted guinea pigs, perfused with buffer containing 0.5% of BSA	TDGA infusion (10 μM, precomplexed with BSA) abolished gluconeogenesis from lactate and pyruvate and production of BHB and acetoacetate. Gluconeogenesis from glycerol and propionate not inhibited. Octanoate addition rescues the rate of ketogenesis but not of gluconeogenesis, unless added before the substrates.	43

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Table 4. Continued

compd	compd name	type of assay	biological matrix	assay conditions	efficacy	ref
		tissue and ex vivo	rat soleus muscle	Determination of $^{14}CO_2$ production from D-[U-^{14}C]palmitate and [1-^{14}C]oleate	0.1 mM TDGA completely inhibited CO_2 production from palmitate, $ED_{50} < 10\ \mu M$. No effect on oleate oxidation. Significant stimulation of glucose oxidation by 0.1 mM TDGA (139−163%) in presence or absence of palmitate. Same observations in soleus muscle of rats pretreated with TDGA (10 (mg/kg)/day for 3 days).	73
rac-**1c**	TDGA-CoA	subcellular	rat liver mitochondria	Medium including 5 mM malonate, 0.5 mM L-carnitine, 10 mg/mL BSA, 20 μM palmitoyl-CoA. Spectrophotometric readout (DTNB)[a]	$IC_{50} = 10\ \mu M$	72
			SD rat liver mitochondria	Formation of palmitoyl[^{3}H]carnitine from palmitoyl-CoA and [^{3}H]carnitine. Conditions as in ref 28 plus ATP and CoA in reaction media and preincubation time of 5 min[b]	$IC_{50} = 0.026\ \mu M$. (*R*)-isomer ~1.7 times more potent than racemate. K_i for irreversible binding of (*R*)-isomer: 0.27 μM.	51
			rat liver mitochondria	Polarographic measurement of oxygen uptake from mitochondria in the presence of palmitoyl-CoA (20 μM) and L-carnitine (0.5 mM)	$IC_{50} = 10\ \mu M$	58
			rat muscle and liver mitochondria	Radiometric assay using [^{14}C]carnitine	IC_{50} = 0.5 and 0.06 μM	65
rac-**1b** or **2b**	POCA or TDGA	tissue	perfused rat heart	Single sc dose of POCA (15 mg/kg) or TDGA (5 mg/kg) 120 min prior to start of the perfusion	Both compounds act in a qualitatively and quantitatively similar way. Reduction of lactate and creatine kinase released in the perfusate, increase in coronary flow by 50%. After total ischemia (30 min) and reperfusion, delay of recovery in the treated heart but improvement of maximum recovery of pressure−rate product 20 min after reperfusion. Reduction of ATP production during ischemia but higher ATP concentrations during reperfusion.	80
rac-**2b**	POCA	cellular	human fibroblasts	Radiometric measurement of $^{14}CO_2$ production from [U-^{14}C]palmitate, [1-^{14}C]pyruvate, and [U-^{14}C]glucose. POCA 5 or 50 μM.	Preincubated cells: 90−93% inhibition of palmitate oxidation, 24−40% increase in pyruvate oxidation, and 250−500% increase in glucose oxidation.	58
			hepatocytes from fed or fasted rats	Measurement of oxidation of 0.5 mM [U-^{14}C]palmitate and production of acetoacetate and BHB.	In fasted rat hepatocytes, $IC_{50} = 5-20\ \mu M$	57
		tissue	perfused livers from fasted (24 h) SD rats	Lactate 1.6 mM, pyruvate 0.2 mM, [U-^{14}C]palmitate 0.2 mM (2:1 with albumin), octanoate 0.5 mM, POCA 10 μM	By addition of POCA to the perfusate: (a) immediate decrease in BHB and acetoacetate production; (b) at 1−2 min, decrease in $^{14}CO_2$ production from [U-^{14}C]palmitate; (c) at 3 min, decrease in glucose production. All effects reversed by infusing octanoate. All processes unaffected by POCA if palmitoyl carnitine is used instead of palmitate.	76
			perfused rat heart	Single ip dose of POCA (30 mg/kg) before surgery to fed or fasted (18 h) rats	Reduction of lipid oxidation in the POCA treated hearts and increase in glucose uptake (2- to 2.5-fold) in both fed and fasted conditions	84
			perfused rat heart	Albumin (3%), palmitic acid (1.2 mM) glucose (5.5 mM), POCA (0.5 mM)	Three protocols: 85 min perfusion; 10 min perfusion + 90 min ischemia; 10 min perfusion + 90 min ischemia + 15 min reperfusion. No effet of POCA on normal working heart (pressure development, cadiac output, coronary flow). Prevention of LCAC accumulation during ischemia. In ischemia−reperfusion, POCA treatment showed faster and more significant recovery of LVDP and cardiac output.	83

Table 4. Continued

compd	compd name	type of assay	biological matrix	assay conditions	efficacy	ref
		ex vivo	rat liver mitochondria	Mitochondria isolated from animals treated with 0.05% or 0.2% POCA food-admix for 12 weeks. Polarographic measurement of oxygen uptake from mitochondria in the presence of palmitoyl-CoA (20 μM) and L-carnitine (0.5 mM).	Up to 80% reduction in oxidation rate	58
			rat muscle mitochondria	Mitochondria isolated from animals treated with 0.05% or 0.2% POCA food-admix for 12 weeks. Polarographic measurement of oxygen uptake from mitochondria in the presence of palmitoyl-CoA (20 μM) and L-carnitine (0.5 mM).	Up to 50% reduction in oxidation rate	58
rac-**2c**	POCA-CoA	subcellular	rat liver mitochondria	Polarographic measurement of oxygen uptake from mitochondria in the presence of palmitoyl-CoA (20 μM) and L-carnitine (0.5 mM)	IC_{50} = 0.3 μM	72
			rat muscle mitochondria	Mitochondria isolated from STZ rats, 48 h fasted. CPT1 activity assayed radiochemically (formation of palmitoylcarnitine)	IC_{50} = 3 μM	56
			rat muscle and liver mitochondria	Radiometric method with [^{14}C]-carnitine	IC_{50} = 0.010 and 0.015 μM	65
			rat liver mitochondria from fed or fasted rats	Radiometric method with [^{14}C]-carnitine	IC_{50} = 0.02 μM in fed rat mitochondria. IC_{50} = 0.06 μM in 24 h fasted rat mitochondria. IC_{50} = 0.1 μM in 48 h fasted rat mitochondria.	76
3		subcellular	rat liver mitochondria	Polarographic measurement of oxygen uptake from mitochondria in the presence of palmitoyl-CoA (20 μM) and L-carnitine (0.5 mM)	IC_{50} = 0.03 μM	72
4		subcellular	rat liver mitochondria	Polarographic measurement of oxygen uptake from mitochondria in the presence of palmitoyl-CoA (20 μM) and L-carnitine (0.5 mM)	IC_{50} = 1.8 μM	72
5		subcellular	rat liver mitochondria	Polarographic measurement of oxygen uptake from mitochondria in the presence of palmitoyl-CoA (20 μM) and L-carnitine (0.5 mM)	IC_{50} = 0.03 μM	58
rac-**6a** or single enantiomers	etomoxir ethyl ester[c]	cellular	rat hepatocytes	Rats treated for 3days with etomoxir before isolating hepatocytes.	Oleic acid (0.4 mM) induced reduction of ^{125}I-insulin and insulin stimulated ^{14}C-AIBA transport by 40%. Etomoxir treatment prevented this effect.	251
			rat hepatocytes	Radiometric measurement of fatty acid synthesis from [2-^{14}C]sodium acetate and of cholesterol synthesis from [2-^{14}C]mevalonate	Inhibition of fatty acid synthesis, $IC_{50} \approx 2$ μM; inhibition of cholesterol synthesis. FAS activity not affected. Both isomers have equal effects.	101
			leukemia cells (OCI-AML3 or MOLM13) alone or on feeder mesenchymal cells	Increasing concentration of etomoxir (0−200 μM) for 96 h	Dose-dependent reduction of viable cell number, IC_{50} = 51−64 μM, via growth reduction and proapoptotic effects. Sensitization to the apoptotic effect of ABT-737 and nutlin **3a**.	214
		tissue	perfused rat hearts	Global ischemia (25 min) followed by reperfusion with 1.2 mM palmitate and 11 mM glucose, to elicit lipotoxicity.	Low dose etomoxir (10 μM) decreased long-chain acylcarnitine and long-chain acyl-CoA levels but did not prevent depressed function. High dose etomoxir (10 μM) prevented the palmitate-induced depression of function but did not decrease long-chain acylcarnitine or long-chain acyl-CoA levels. In aerobic hearts not subjected to ischemia, 10 μM etomoxir increased glucose oxidation both in the presence and absence of palmitate. Etomoxir cardioprotection is unrelated to levels of long-chain acylcarnitines.	85

Table 4. Continued

compd	compd name	type of assay	biological matrix	assay conditions	efficacy	ref
			perfused rat hearts from acute (48 h) and chronic (6 weeks) STZ-diabetic rats	Partial ischemia (50%) followed by reperfusion, in the presence or absence of palmitate (1.2 mM)	Addn of 1.2 mM palmitate to the perfusate accelerated failure rates, which were more evident in chronic than in acute diabetic rats. In chronically diabetic rats, mechanical function could not be maintained in palmitate-perfused hearts, even in the absence of ischemia. 0.01 μM etomoxir markedly decreased the rate of mechanical failure in both acutely and chronically diabetic rat hearts, in the presence and absence of palmitate independent of long-chain acyl-CoA or long-chain acylcarnitine levels.	88
rac-**6b**	etomoxir	cellular	bovine aortic endothelial cells	5 mM glucose, 800 μM oleic acid, 1 mM albumin, with or without TDGA (concentration not specified)	Complete reversal of FFA-induced ROS production, GAPDH down-regulation, PKC up-regulation, GlcNAc-modified protein up-regulation, PGI2 synthase activity down-regulation, nitrated PGI2 synthase up-regulation, eNOS activity down-regulation.	252
rac-**6c**	etomoxir-CoA	subcellular	rat heart mitochondria	Radiometric assay using [1-^{14}C]palmitate	IC_{50} = 0.009 μM with preincubation. IC_{50} > 0.10 μM without preincubation. Several data on CAT and COT reported.	65
			rat muscle and liver mitochondria	Radiometric assay using [^{14}C]carnitine	IC_{50} = 0.025 and 0.003 μM	65
		isolated enzyme[d]	intact mitochondria preparation from yeast *Saccharomyces cervisiae* expressing rat enzymes	Radiometric method with [methyl-^{3}H]carnitine	IC_{50} = 0.70 μM for CPT1A and 0.04 μM for CPT1B	70
rac-7-CoA	DET-CoA	subcellular	rat muscle and liver mitochondria	Radiometric assay using [^{14}C]carnitine	IC_{50} = 0.010 and 0.012 μM	65

[a] These derivatives cannot be assessed in isolated enzyme assays, as they cannot be converted to the -CoA derivatives. Moreover, thiol-reactive agents like DTNB are not adequate to measure malonyl-CoA sensitivity of CPT1A or CPT1B.[180] This constitutes a limitation of the assay. For this reason, the -CoA derivatives of oxirane carboxylic acids and **55** could not be assessed in this assay.[181] [b] Prepared either chemically or enzymatically. [c] The exact molecular species used is at times not explicitly mentioned in the literature. As the name "etomoxir" generally defines the racemic ethyl ester, it is assumed in ambiguous cases that this is the compound used. [d] The structure of etomoxir reported by these authors does not correspond to that reported in the bulk of the literature.

Table 5. Summary of in Vivo Pharmacological Assays Performed with Oxirane Carboxylic Acids

compd	compd name	species	route	dose	observations	ref
rac-**1a**	Me-TDGA	db/db mice	po	50−100 mg/kg	Lowering of blood glucose 2−4 h postdose, −40% in fed and −78% in fasted state.	46
		SD rats	po	2−150 mg/kg	1 h postdose, dose-dependent reduction of glucose excursion in subcutaneous GTT[a]	46
			po	5−20 mg/kg	7 h postdose, dose-dependent reduction of fasting blood glucose	46
			po	25 mg/kg	In fasted animals, inhibition of FAO, stimulation of glucose oxidation, elevation of plasma FFAs, lowering of blood glucose, depletion of muscle and liver glycogen	47
			po	10−20 mg/kg	In fasted animals, [3-^{14}C]-pyruvate ip injection 1−8 h postdose: considerable depression (∼50%) of conversion of labeled pyruvate to [^{14}C]-glucose	48
			po	0.05−10 mg/kg	In fasted animals, dose dependent lowering of plasma KB starting from 0.2 mg/kg, correlating with reduction of CPT activity in isolated mitochondria from treated animals In fed animals, no effect on glucose and KB, although CPT activity equally reduced.	75
		HFD Wistar rats	po	10 (mg/kg)/day for 14 days	Hypoglycemia, depletion of muscle and liver glycogen, increase in plasma FFA	78
		STZ rats	po	10−50 mg/kg or 10 (mg/kg)/day for 2 days	In 16 h fasted rats, lowering of blood glucose. In fed, severely hyperglycemic rats, lowering of blood glucose.	46
			po	2 × 20 mg/kg	In fasted animals, inhibition of FAO, doubling of glucose oxidation, lowering of blood glucose, no effect on plasma FFAs	47
		alloxan-treated rats	po	25−50 mg/kg	70−100% lowering of KB levels, 2−5 h postdose	46
			po	0.2−2.5 mg/kg	Decrease in CPT activity in mitochondria isolated from treated animals, similar to nondiabetic controls. Normalization of KB.	75
		nondiabetic Beagle dogs	po	20−100 mg/kg	In fasted dogs, delayed hypoglycemia with increase of FFAs. No effect in fed animals.	46
		depancreatized Beagle dogs	iv	20 mg/kg	1−7 h postdose, normalization of plasma and urine glucose, ketonuria, and ketonemia. No effect on FFAs, TG, and insulin.	46
			po	20−50 (mg/kg)/day for 5 days	Lowering of blood ketone bodies and plasma and urine glucose in the fasting state. No effect on glucose excursion after feeding.	46
		STZ or STZ + alloxan treated dogs	po	2.5 (mg/kg)/day for 7 days	After 6 days, lower overnight fasting glucose (−8%). No difference from vehicle in recovery from insulin-induced hypoglycemia, due to unhindered increase in HGP.	187
		STZ or alloxan treated dogs	po	0.7−2 (mg/kg)/day for 7 days or 7.5 mg/kg single dose	Reduction of blood glucose from 9 to 6.5 mM 6 h postdose (overnight fasting)	74
		human	po		One diabetic patient with anti-insulin receptor antibodies. Me-TDGA treatment resulted in a decrease in plasma glucose concentration (from 24 to 16 mM) associated with a decrease in the rate of glucose production due to decreases in both gluconeogenesis and glycogenolysis rates. Patient died of hypoglycemic coma after 2 months.	90
			po	50 mg/day for 11 days (study 1) and 50 mg/day for 28 days (study 2)	In study 1, six ambulatory ketotic T1D patients, treated on top of insulin, exhibited immediate and sustained disappearance of ketonuria and reduction of glycosuria, FBG, and postprandial blood glucose, three patients required reduction of insulin dosage. In study 2, 7/9 nonketotic T1D patients treated on top of insulin exhibited reduction of glycosuria and plasma glucose levels. No effect on BW, routine hematology, biochemical parameters, and ECG.	212
rac-**1b**	TDGA	C57BL/6J mice	ip	30 mg/kg	12 h postdose, in the fasted state, severe microvescicular liver steatosis and elevation of plasma FFAs and TGs. VLDL-TG production rate not affected and was suppressed normally by insulin. IRS1- and IRS2-associated PI3 kinase activity and PKB phosphorylation were not affected.	248
		db/db mice	po	5, 10, and 25 (mg/kg)/day for 12 weeks	No effect on food intake or growth. Reduction in blood glucose 4 h postdose in the middle and end portion of the study. No effect on plasma FFAs. No liver lipid deposition. Significant inhibition of immunopathological changes in the kidney (diabetic nephropathy) in the 25 mg/kg group. Significant increase in heart weight in the 25 mg/kg group.	48, 44

Table 5. Continued

compd	compd name	species	route	dose	observations	ref
		SD rats	po	2.5−60 mg/kg	In fasted animals (18−24 h), reduction of glucose excursion in scGTT, ED_{50} = 6 mg/kg. Hypoglycemia and death at ≥30 mg/kg.[b]	78
			po	0.25−5 mg/kg q.d. or b.i.d. for 3 days	In fasted animals, reduction of blood glucose at doses of ≥0.5 mg/kg b.i.d. and ≥1 mg/kg q.d.	78
		swine	po	68.3 mg/kg for 3 days	Study on cardiac function during and after simulated ischemia. TDGA treatment reduced $^{14}CO_2$ production from [U-^{14}C]palmitate infused directly in the heart under aerobic conditions (<10% of control) but not under ischemia. Tissue levels of acyl-CoA and acylcarnitines were reduced. Improvement of mechanical function during aerobic phase, but no difference with vehicle during ischemia.	82
		guinea pigs	po	10−25 mg/kg	In 72 h fasted animals, marked decrease of KB and 59% decrease of plasma glucose at 10 and 25 mg/kg. No effect in 24 or 48 h fasted animals.	43
rac-**2a**	POCA ethyl ester	SD rats	po	2.5−600 μmol/kg (=0.7−178 mg/kg)	Lowering of blood glucose in fasted animals, max decrease at 600 μmol/kg = 80%, ED_{50} = 6 mg/kg, LD_{50} = 275 mg/kg (hypoglycemic coma); pronounced decrease of blood KB	55[c]
rac-**2b**	POCA	db/db mice	po	190 μmol/kg (=55.3 mg/kg)/day for 4 days	High interindividual variability in FBG (−60% 1 h postdose, 16 h fast). OGTT in fasting state, with last dose 1 h before glucose load: ∼50% reduction of AUC.	54
		SD rats	po	2.5−600 μmol/kg (=0.7−178 mg/kg)	Lowering of blood glucose in fasted animals, max decrease at 600 μmol/kg = 80%, ED_{50} = 9 mg/kg, LD_{50} = 486 mg/kg (hypoglycemic coma); pronounced decrease of blood KB	55
			po	0.6−60 μmol/kg (=0.16−16 mg/kg	4 h postdose. In fed state, no effect on blood glucose despite up to 99% enzyme inhibition (demonstrated by determination of CPT1 activity in liver mitochondria from treated animals in parallel to glucose measurement). Effect on blood KB (up to 40% reduction). In fasted state, up to 25% blood glucose reduction and 78% KB reduction, parallel to enzyme inhibition (up to 98%).	76
			po	8−300 μmol/kg (=2.3−87 mg/kg)	In fasted (16 h) rats 2 h postdose, decrease of blood glucose up to 50%, lasting for 12 h. ED_{50} (25%) = 8.7 ± 3 mg/kg. Blood KB lowering (especially BHB) up to 75%. Dose dependent elevation of plasma FFAs, ∼300% at the lowest dose 2−4 h postdose. Dose dependent elevation of plasma TG (up to 290%). Decrease in total cholesterol 24 h postdose (−43%). No effects in fed rats. Similar observations after iv administration.	54
		adrenalectomized rats	po	25 μmol/kg (=7.25 mg/kg) and 150 μmol/kg (=43 mg/kg)	34% decrease in blood glucose at the lower dose, death of all animals at the higher dose (hypoglycemic coma). Increase in plasma FFAs and decrease in KB (with decrease in the ratio of 3-OH-butyrate/acetoacetate.	54
		guinea pigs (SPF-white inbred)	po	7−56 μmol/kg (=2−16.3 mg/kg)	In fasted animals (48 h), similar effects as in rats on blood glucose, ED_{50} (25%) = 8.7 ± 3 mg/kg, but different kinetics (effect short lasting, max effect at 2 h, baseline at 5 h).	54
		Beagle dogs	po	100 μmol/kg (=29.1 mg/kg)	In fasted animals (24 h), rapid lowering of blood glucose up to 4 h postdose	54
		STZ diabetic pigs (Hanford miniature)	iv	10−40 μmol/kg (=2.9−11.6 mg/kg)	Up to 35% decrease of blood glucose 6 h postdose	54
rac-**6a**	etomoxir ethyl ester[d]	mice	icv	0.032 and 0.320 μmol once	Increase of food intake 0.5−1 h postdose, weight loss on day 1 at the highest dose, followed by hyperphagia and increase in body weight in the subsequent days	175
		nude mice xenotransplanted with GFP/luciferase bearing MOLM13 human leukemia cells	ip	50 mg/kg every second day for 3 weeks	In combination with ABT-737, treated mice had significantly lower leukemia burden than with ABT-737 alone and prolonged survival rate (33%), while neither ABT-737 nor etomoxir alone prolonged survival. Even stronger effect of etomoxir in combination with Ara-C	214
		db/db mice	po	10 and 50 (mg/kg)/day for 74 days	Dose dependent reduction of FBG level of 43% and 55%. Total abolition of glucosuria.	96
			po	10 or 20 (mg/kg)/day life-long, food-admix.	Prolongation of life expectancy. Reduced fasting glucose, reduced daily water intake (∼50%), urine volume, and glucose excretion (∼−70%)	45
		SD rats	icv	0.58 μmol once	22 h after injection, decrease of cumulative food intake (starting 4 h postdose) and body weight	172
		STZ treated rats	po	0.2−18 mg/kg	In fasted animals, dose dependent decrease of blood glucose 4 h postdose (−40%), dose-dependent decrease of KB (−87%), in parallel with decrease in CPT1 activity (up to 99% decrease)	96

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Table 5. Continued

compd	compd name	species	route	dose	observations	ref
			sc	12.5 and 25 (mg/kg)/day for 3 weeks	Significant reduction of fasting plasma glucose (−32% to −40%) and hepatic glucose production (−32 to −35%). No increase in FFAs or plasma TG. Isolated hepatocytes from treated rats manifest enhanced sensitivity to insulin.	196
		STZ-treated rats, on a high fat diet	sc	12.5 (mg/kg)/day for up to 4 weeks	Reduction of fasting plasma glucose, fasting plasma TG (−67) and fasting serum FFAs (−56%), already evident after 3 days to 1 week of treatment	215
		STZ rats	po	50 mg/kg with or without concomitant niacin (200 μmol/kg sc at 0, 60, and 120 min)	Synergistic effect with niacin in lowering blood glucose. Niacin prevents the rise in TG and FFA concentration due to etomoxir.	254
		normal and chronically diabetic rats.	ip	18 (mg/kg)/day for 6 days	Amelioration of decreased heart performance in diabetic rats. Reduction of serum concentration of glucose, TG, cholesterol. Increase in heart lipid and carnitine content.	86
		Wistar rats with ascending aorta constriction	po	15 (mg/kg)/day for 12 weeks	Slight hypertrophic growth in right and left ventricles of sham-operated rats and in right ventricles but not in overloaded left ventricles of rats with aortic constriction. Increase of maximal developed pressure, left ventricular pressure−vol area, and $\pm dP/dt_{max}$ in all treated rats. Significant increase of myosin V1 in pressure-overloaded left ventricles.	89
		human	po	50 mg	Strong reduction of KB after 18−36 h of fasting, glucose reduction (−40%) after 36 h of fasting, with concomitant increase of lactate and pyruvate (inhibition of gluconeogenesis)	41
			po	125 mg/day for 4 days	Lowering of postprandial glucose from 10.5 to 8.3 mM in NIDD patients	41
			po	50, 100, 150, 200 mg/day (b.i.d. dosing) for 3 days before meals (morning and evening) in eight obese NIDD patients	Decrease of fasting plasma glucose by 18%. Reduction of basal hepatic glucose production by 18%. No effect on glucose utilization in euglycemic hyperinsulinemic clamp and no effect on glucose oxidation rate (indirect calorimetry). Reduction of basal plasma insulin concentration. Decrease in BHB by 44%. Decrease in plasma cholesterol by 24% and of TG by 54%. Increase in plasma FFAs by 52%. No effect on total lipid oxidation. Doubling of plasma GPT and ALT concentration.	95
			po	12.5, 25, and 50 mg/day for 14 days in 48 NIDD patients	Reduction of FBG up to 45%, reduction of β-hydroxybutyrate by 50%. Reduction of plasma lipids (TG, −30%; LDL/HDL, −35%). Increase of GPT 2-fold by some patients.	96, 94
			po	25 mg	Turnover study with 6,6-D_2-glucose, decrease of gluconeogenesis without effect on peripheral glucose utilization	41
			po	50 mg once in NIDD patients	Euglycaemic clamp in NIDD patients. Increase in metabolic clearance of glucose by 30%.	96
			po	100 mg/day for 3 days	Euglycaemic clamp in NIDD patients. Increase in carbohydrate oxidation by 58%, decrease in fat oxidation by 18%, decrease in glucose appearance rate by 8%. Decrease in nonoxidative glucose utilization. No effect on glucose infusion rate. Study was run with isotope dilution MS (6,6-D_2-glucose) and indirect calorimetry.	97
			po	75 or 150 mg once	In subjects with habitually high fat intake, increase of hunger feeling. Decrease in plasma β-hydroxybutyrate, increase in plasma FFA and lactate.	91
			po	320 mg once	In subjects deprived of time cues, no effect of etomoxir on any variable of satiety. Increase in FFA and decrease of BHB.	224
			po	600 mg etomoxir total over 5 days in subjects previously fed a high fat diet for 3 days	Significant increase in respiratory quotient in the treated group, decrease in whole body fat oxidation (−13.7%). Positive fat balance and negative carbohydrate balance. No effect on hunger and satiety.	92
			po	50 mg once in NIDD patients	Euglycemic clamp revealed a significant increase in MCR of glucose by 33%. 3/8 patients, however, did not respond to treatment. Increase in MCR seems to correlate negatively with FFA and TG increase.	98
			po	80 mg/day for 3 months in CHF patients	Increased maximum cardiac output during exercise (+38%), increase in stroke volume (+30%), increase in left ventri-cular ejection fraction (+25%). No acute effects on heart function. No acute inotropic or vasodilatatory effects.	204
			po	40 and 80 mg/day for 6 months (ERGO study)	Study terminated prematurely because of ALT and AST increase in four patients in the treatment groups. Trend toward increase in exercise time in the treated groups.	208

Table 5. Continued

compd	compd name	species	route	dose	observations	ref
rac-**6b**	etomoxir	BNX mice with skin transplants from psoriatic donors	topical	1% and 30% etomoxir emulsified in wool wax, Vaseline, and cetylstaryl alcohol	Significant reduction of dermal thickness, decrease of Ki-67+ cells and ulex-positive cells, similar to the effects of betamethasone (positive control). Effects were dose-dependent.	213
		lean and obese mice (via ip injection of gold thioglucose)	po	25 mg/kg	OGTT after an overnight fast showed reduction of AUC for both the lean and obese mice (1 h postdose). Reduction of serum insulin in fasted mice. Significant increase of pyruvate dehydrogenase complex activity in heart, WAT and liver but no increase in quadriceps muscle.	197
		DIO mouse	ip	9 (mg/kg)/day for 7 days	PGI2 synthase activity and eNOS activity in aorta restored to normal (standard diet control) levels	252
		fa/fa rats	ip	18 (mg/kg)/day for 7 days	PGI2 synthase activity and eNOS activity in aorta restored to normal (fa/fa control) levels. Confirmation that this is not an effect of eNOS overexpression.	252
		Wistar rats	iv	60 mg/kg over 5.5 h	Kinetic studies with [U-^{14}C,3-^{3}H]glucose. Decrease in plasma glucose by 25%, decrease in whole body glucose utilization by 14%, decrease in recycling of glucose carbon by 30%. Dramatic lowering of blood 3-OH-butyrate. from 1 mM to ~0.03 mM.	195
			po	Food-admix containing 0.1% **1** for 10 days, followed by bolus infusion of 150 mg/kg over 5.5 h	No effect of acute infusion on plasma glucose but increased glucose turnover and recycling of glucose carbon by 40% (chronic, not acute effect). Increase of pyruvate dehydrogenase in quadriceps muscle.	195
		pigs	iv	20 mg	In 48 h fasted animals, lowering of plasma glucose by 50% 5 h postdose. Kinetic studies with [U-^{14}C,3-^{3}H] glucose reveal that this is due to increase in glucose utilization in the muscle.	194

[a] The free acid **1b** was also found to reduce glucose excursion in a similar experiment but with lower efficacy, probably because of poorer oral absorption. [b] Activity was shown to persist up to 10 h postdose. [c] This reference reports in vivo activity of a number of simple derivatives of POCA, which are all consistently less potent. [d] When the exact molecular species used is not explicitly mentioned in the paper, it is assumed that this is the compound used in the study, as the name "etomoxir" generally defines the racemic ethyl ester.

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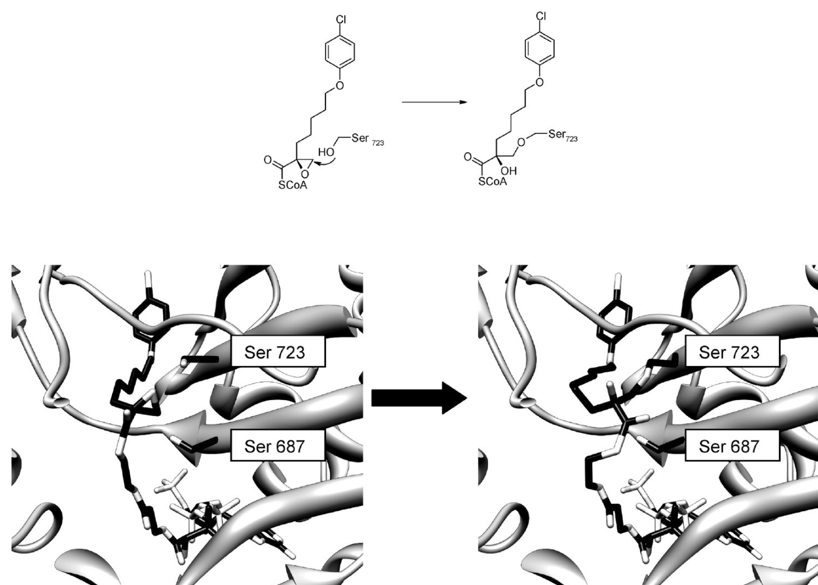

Figure 4. Putative mechanism of the covalent reaction between etomoxir-CoA and human CPT1B. The homology model of hCPT1B is based on the known X-ray structure of hCPT2. The structure of bound CoA is based on the CAT X-ray 1ndi.

compounds **6a**,**b** and their derivatives have been published. Most derivatives of **6** are reported in the patent literature, with a corresponding lack of biochemical characterization which makes it difficult to judge the relevance of the compounds for CPT1 inhibition. The dechlorinated analogue **7** appears to have similar potency and selectivity as **6**.[65] The dinitro derivative **8** (DNP-Et) is relatively well characterized and reported to be a selective inhibitor of CPT1A over CPT1B (maximal inhibition at 5 μM vs $\gg$60 μM, respectively, in rat liver and muscle mitochondria). This compound was used to characterize the relative contribution of CPT1A vs CPT1B to cardiac FAO control.[66] An α-fluorinated derivative (**9**) is reported to have selectivity for CPT1A over CPT1B (IC_{50} of 0.25 μM vs >50 μM).[67] Such compounds, including also derivatives of **2** such as compound **10**, were also described as phospholipase A2 inhibitors (IC_{50} = 2.6 μM for **10**).[68] Bifunctional compounds including the oxirane carboxylic acid structure attached to either 2-deoxy-D-glucose or hypoglycin A were reported to be dual FAO and glycolysis inhibitors, with potential in cancer treatment.[69]

All oxirane carboxylic acids and their esters share not only a similar chemotype but also a very similar mode of action. All are prodrugs, which are essentially inactive in membrane preparations expressing any of the recombinant enzyme isoforms (see Table 9). The esters are water insoluble oils but manifest oral bioavailability. The acids and their sodium salts are water-soluble and have been used for both the oral and the parenteral route. The esters are converted to the acids in vivo, and these need to be converted to the CoA thioesters by acyl-CoA synthetase to be efficacious. This double activation step and the irreversible nature of the inhibition (see below) obviously complicates the relative assessment of the potency and specificity of oxirane carboxylic acid and their esters. A further element of complication is a significant difference in oxidation rates and sensitivity to the CPT1 inhibitor etomoxir between hepatocytes of different species, with human hepatocytes showing the lowest and rat hepatocytes the highest rate of β-oxidation with an opposite trend for sensitivity to etomoxir ($\sim$100-fold difference in IC_{50}).[22]

The CoA thioesters of oxirane carboxylic acids are covalent irreversible inhibitors of the CPT enzymes, although the nature of the covalent bond formed was never fully demonstrated. Contrary to what was reported in a recent paper,[70] the bulk of the literature dictates that the species formed by reaction of the oxirane carboxylic acids with CoA is the thioester and not the product of nucleophilic substitution at the epoxide.[48,49,71,72] The epoxide moiety of the CoA ester might then be involved as an electrophile in a ring-opening reaction. From modeling studies with a homology model of CPT1B based on the CPT2 crystal structure, it appears plausible that the amino acid residue involved in the formation of this covalent bond in human CPT1B is Ser(723). Ser(687), which also appears possible, has a less favorable distance and orientation to participate directly in the epoxide ring-opening, although it might be involved in stabilization of the transition state. Figure 4 shows the putative mechanism for this reaction, with the serine nucleophile attacking the epoxide in a S_N1-like fashion. This mechanism also explains why oxirane carboxylic acids are selective CPT1 inhibitors. As shown in the sequence alignment of the relevant region, reported in Figure 5, while Ser(723) is conserved throughout the CPT 1 series, in CPT2 this is substituted by either Ala or Gly, depending on the species, so that the covalent bond with the epoxide cannot form.

In vivo, all oxirane carboxylic acids strongly promote hypoketonaemia in fed and fasted normal animals (mice, rats, guinea pigs, dogs, and pigs) and humans but only affect glucose levels significantly in the fasted state (see Table 5). Toxicity is also observed mainly in fasted animals. In streptozotocin- or alloxan-treated animals (a model of type 1 diabetes), glucose may or may not be lowered, while ketonemia may return to normal values. The effective doses of the oxirane carboxylic acids vary greatly depending on the species, the nutritional state, and the length of the treatment. All oxirane carboxylic acids, with the possible exception of **8**, are nonselective CPT1A/CPT1B inhibitors, whereas no activity at CPT2 is observed. The peripheral effect of these compounds in limiting FAO oxidation in the muscle and therefore promoting glucose utilization certainly plays a role in

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Q8BGD5  AHDHGYGISYIFMGENAITFHISSKKSSTETDSHRLGQHIENALLDVASLFRVGQHFKRQ  769  CPT1C_MOUSE
Q8TCG5  ADDHGYGVSYIFMGDGMITFHISSKKSSTKTDSHRLGQHIEDALLDVASLFQAGQHFKRR  771  CPT1C_HUMAN
P32198  VADDGYGVSYIIVGENFIHFHISSKFSSPETDSHRFGKHLRQAMMDIITLFGLTINSKK-  773  CPT1A_RAT
P97742  VADDGYGVSYIIVGENFIHFHISSKFSSPETDSHRFGKHLRQAMMDIITLFGLTANSKK-  773  CPT1A_MOUSE
P50416  VADDGYGVSYILVGENLINFHISSKFSCPETDSHRFGRHLKEAMTDIITLFGLSSNSKK-  773  CPT1A_HUMAN
Q68Y62  VADDGYGVSYILVGENLINFHISSKFSSPETDSHRFGKHLKQAMNDIMALFGFSSNSRKE  774  CPT1A_HORSE
Q8HY46  VADDGYGVSYMIAGENTIFFHVSSKFSSSETNAQRFGNHIRQALLDLADLFQVPKTDS--  772  CPT1B_PIG
Q58DK1  VADDGYGVSYMIAGENTIFFHVSSKFSSSETNAQRFGNQIRQALLDIANLFQVPKADG--  771  CPT1B_BOVIN
Q92523  VADDGYGVSYMIAGENTIFFHISSKFSSSETNAQRFGNHIRKALLDIADLFQVPKAYS--  772  CPT1B_HUMAN
Q63704  VADHGYGVSYMIAGENTMFFHVSSKLSSSETNALRFGNHIRQALLDIADLFKISKTDS--  772  CPT1B_RAT
Q924X2  VADDGYGVSYMIAGENTMFFHISSKYSSSETNAQRFGNHIRQALLDIAELFKISKTDS--  772  CPT1B_MOUSE
P23786  VVSDGFGVGYAVH-DNWIGCNVS---SYPGRNAREFLQCVEKALEDMFDALEGKSIKS--  658  CPT2_HUMAN
Q60HG9  VVSDGFGVGYAVH-GNWIGCNVS---SYPGRNAREFLQCVEKALEDMFDALEGKSIKS--  658  CPT2_MACFA
Q2KJB7  VVPDGFGIGYSVQ-DNWIGCNVS---AYQSRNAREFLQCVEKALEDMFDALEGKMIKT--  658  CPT2_BOVIN
P18886  VVPDGFGIAYAVH-DDWIGCNVS---SYSGRNAREFLHCVQKCLEDIFDALEGKAIKT--  658  CPT2_RAT
P52825  VVPDGFGIAYAVH-DDWIGCNVS---SYSGRNAREFLHCVQKCLEDMFDALEGKAIKT--  658  CPT2_MOUSE
Q6P4X5  VVPDGFGVGYGVH-DDWIGCNVS---SYQTRDVRQFVECVHQSLDDIFTVLQDKPIK---  658  CPT2_XENTR
Q7ZXE1  VVPDGFGVGYGVH-DDWIGCNVS---SYPARDVRQFVQCVHQSLDDIFSVLQDKPLK---  659  CPT2_XENLA
Q5U3U3  VVPDGFGVGYGVH-DEWIGCNVS---SYPARDVHEFLRCVHKSLEDIFTVLDGNPIH---  669  CPT2_DANRE
P17898  LVWMGLGLTLAIH-------------------GMFINDIIYDITTFLDIYALSIKHPKE  392  CPT1_YEAST
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Figure 5. Sequence alignment of the oxirane-binding region for various CPT isoforms. The serine involved in the formation of the covalent bond is highlighted. In CPT2 this residue is substituted by Ala or Gly.

their hypoglycemic effect. Compounds **1**, for example, were demonstrated to stimulate oxidation of D-[U-^{14}C]glucose by rat soleus muscle in a dose dependent manner both in vitro and ex vivo.[73] Sherratt et al. postulated that acute oral doses would act preferentially at the liver level, regardless of the isoform specificity, because of a sort of pharmacological "first-pass" effect. Upon chronic treatment, inactivation of CPT1B at the muscle level by these irreversible inhibitors would take an increasingly important role. Thus, diabetic dogs show a very significant decrease of blood glucose after 5 days of treatment with small oral doses of **1a**, which would not be sufficient in themselves to elicit a glucose reduction in an acute experiment.[74] Given the human data, however (see section on therapeutic potential), this most likely reflects the intrinsic preference of **1** for CPT1A vs CPT1B, while **6** probably acts on both axes at the same time. It is important to note that the delay between KB reduction and glucose reduction on the dose and time level is a consistent characteristic of all CPT inhibitors, regardless of the isoform specificity.

Tutwiler et al. attempted to establish a correlation between the degree of CPT1 inhibition and the effects on glucose and ketone bodies in rat.[75] In these experiments, liver, heart, and diaphragm mitochondria were isolated from 48 h fasted rats that had been treated with oral doses of **1a**, and CPT1 and CPT2 activities were measured in these preparations. The results confirmed a higher sensitivity of CPT1A toward inhibition by **1a** compared to CPT1B, while CPT2 was unaffected. CPT1 inhibition by **1a** clearly preceded the observed hypoglycemic and hypoketotic effect. Acute lowering of ketone bodies was obtained at doses 10 times lower than those affecting glucose levels, and the effect was persistent over 48 h, as expected from an irreversible inhibitor. In an interesting study by Wolf and Engel[76] the time delay between inhibition of ketogenesis and inhibition of gluconeogenesis following CPT1 inhibition by **2**, as well as the interdependency between nutritional state, CPT1 activity, KB and glucose levels in vivo, in the rat was demonstrated. In general, with all CPT inhibitors, effects that are a direct consequence of FAO inhibition (increase in circulating FFAs, decrease in KB and other markers, inhibition of palmitate oxidation in ex vivo studies) are evident before any lowering of glucose levels either through increase in glucose utilization or decreased gluconeogenesis and can be measured on both the time and the dose axes (see Figure 6),[78,77] although a clear quantitative correlation has remained elusive.[78]

Some of the experiments with **2** reveal other interesting aspects of the system pharmacology of CPT inhibition. The compounds showed no effect on insulin secretion or plasma insulin concentration in rats or isolated perfused rat pancreas and did not affect the mitochondrial respiratory chain, oxidative phosphorylation, or the citrate cycle, even at very high concentrations. The rate of palmitoyl-CoA hydrolysis appears increased in mitochondria obtained from rat treated chronically with **2b**.[58] The behavior of the FFA level in adrenalectomized rats (which increases to a more limited extent than in normal animals) and the fact that these animals are more sensitive to hypoglycemia upon CPT inhibition point to a counterregulatory effect of catecholamine-induced lypolisis to inhibition of FAO.[54] The effects on STZ-treated pigs[54] indicate potential effects of CPT inhibition in T1D, as documented for **1a**.[212]

Positive effects of oxirane carboxylic acids on heart function have been described with **1** in rat[79−81] and swine[82] and with **2** in rat.[80,83,84] Animal studies on heart function with **6** were performed in rat tissue.[85−89]

Compound **1a** was tested in humans in the context of diabetes,[90,212] and **6b** was involved in a number of clinical studies for metabolic syndrome,[91,92] diabetes,[93−98] and cardiac failure,[99,100] although it never reached the market.

Oxirane carboxylic acids have a rich polypharmacology on top of CPT1 inhibition. Compound **6b** was shown to inhibit fatty acid and cholesterol synthesis via a mechanism that is independent from its effect on inhibition of FAO.[101] Indeed, the (*S*)-isomer of **6b**, which is inactive at CPT, was shown to have the same inhibitory effect on these processes in isolated rat hepatocytes as the (*R*)-isomer, clearly demonstrating a structural and not a mechanistic correlation. Racemic **2b**[57] and racemic **1a**[102] were also shown to inhibit fatty acid synthesis and cholesterol synthesis[103] in isolated hepatocytes. Compounds **6** and their derivatives are PPARα agonists,[104] and it is conceivable that the effects on fatty acids and cholesterol synthesis are secondary to this pharmacology. It has been discussed whether the induction of PPARα-related genes is a direct action of **6** or is secondary to CPT inhibition,[244] and the former appears more likely. This "off-target" effect of **6** has a massive influence on in vivo profiles (see section on therapeutic potential and safety aspects), is likely shared by all other oxirane carboxylic acids (**1b** was also demonstrated to be a PPARα agonist), and has often been underestimated in the literature. As discussed above, **6c** also inhibits

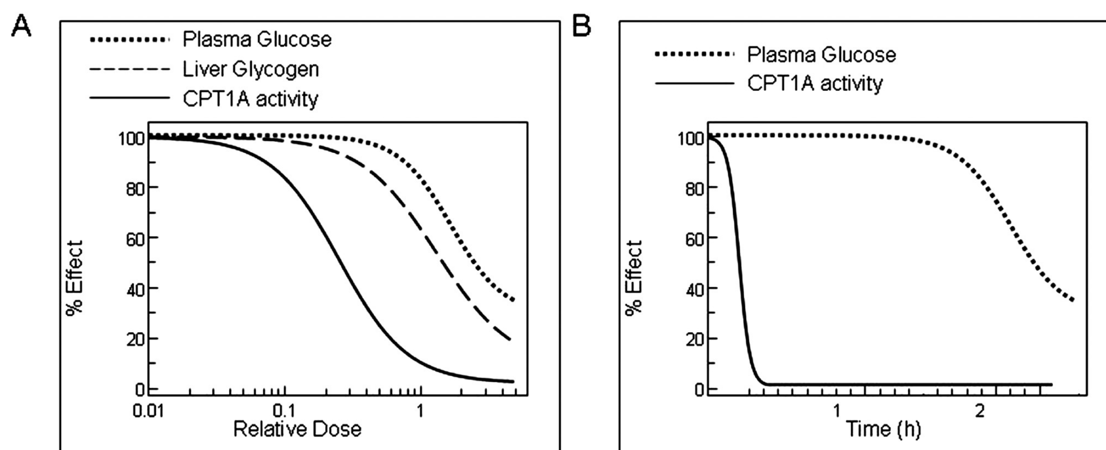


Figure 6. Schematic representation of the behavior of biomarkers of CPT1A inhibition (e.g., plasma acetylcarnitine, KB) vs glycemia biomarkers (e.g., plasma glucose, liver glycogen) on the dose (A) and time (B) axis. Note that although the graphs are based on real data, they do not contain absolute quantitative information on the magnitude of the effects.

COT and CAT, and close analogues have been shown to be phospholipase A2 inhibitors.

Patents pertinent to **1** and analogues,[105] **2** and analogues,[106] **6** and analogues,[107] and related compounds[108] have been filed and are partly expired.

Carnitine Derivatives and Analogues. Along the tradition of using the natural substrates of the CPT enzymes as models for the development of pharmacological agents, considerable attention was also dedicated to carnitine, the second partner of the reaction equation, as well as to analogues of carnitine that would resemble the transition state or the products of the reaction. A more systematic SAR exploration was granted to this set of compounds, and at least two development candidates emerged from these efforts. Scheme 2 reports representative structures of this class of compounds. Tables 6 and 7 report an almost comprehensive summary of most in vitro and all in vivo data generated with this class of compounds. Again, the reader is referred to the original literature for more details. For discussion, the topic will be divided into subclass-related paragraphs.

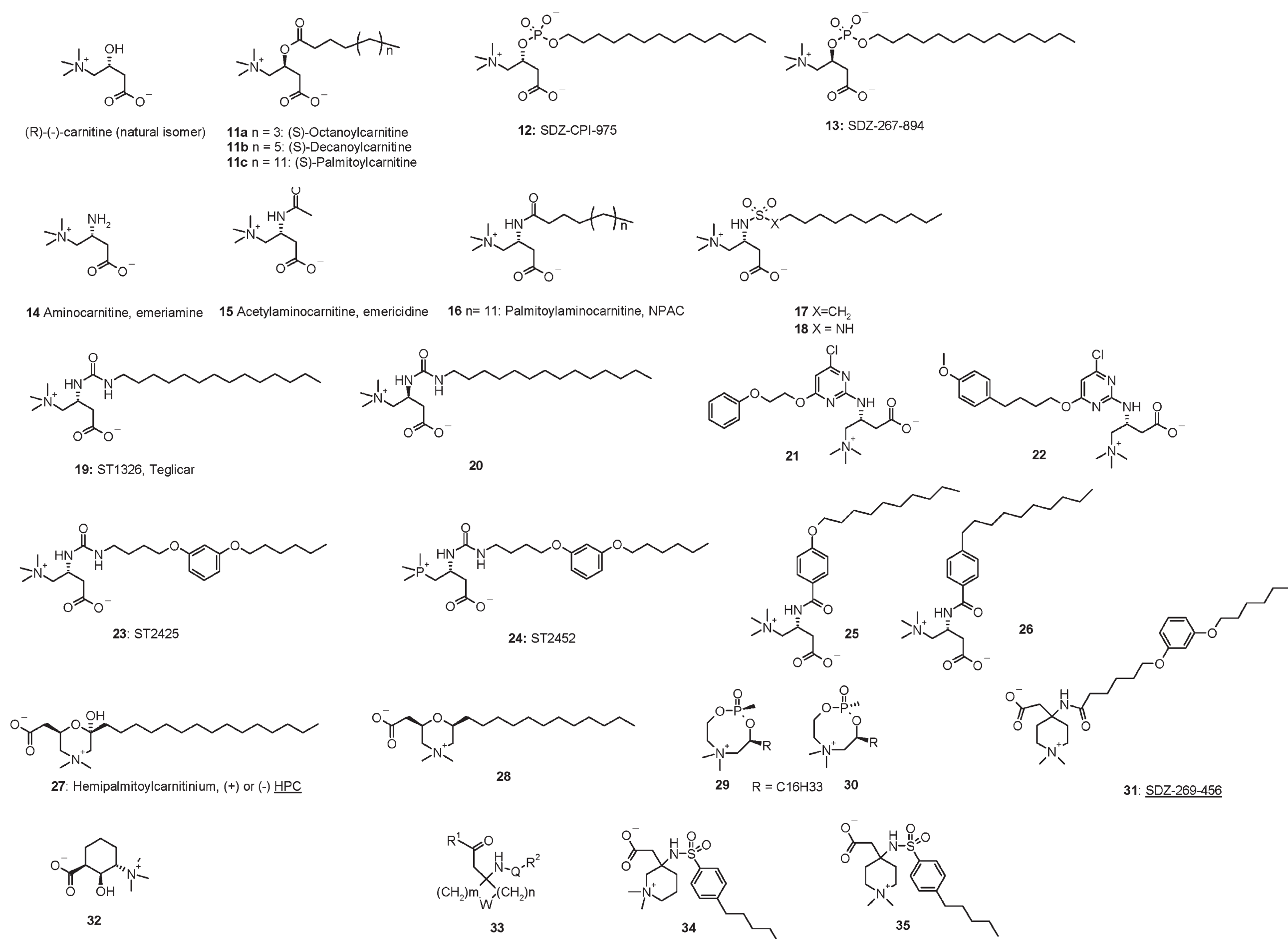
(S)- and (R)-Carnitine Derivatives. Speculating on the use of nonprocessable substrate analogues, acyl derivatives of the unnatural isomer of carnitine, (*S*)-carnitine, have been described as FAO inhibitors at the level of CPT in several papers.[109] Interesting experiments in models of diabetic ketoacidosis in the rat were performed with these compounds, which were shown to have a strong antiketogenic effect and to reverse ketosis much more rapidly and effectively than insulin. (*S*)-Octanoylcarnitine **11a** was particularly effective in these experiments. McGarry et al., however, demonstrated that (*S*)-acylcarnitines are not affecting either of the CPT enzymes but are inhibitors of the carnitine−acylcarnitine transporter CACT, particularly **11a** (IC_{50} = 35 μM), **11b** (IC_{50} = 5 μM), and **11c** (IC_{50} = 35 μM).[110] As this is also a central element of the CPT machinery, it is understandable that (*S*)-acylcarnitines have been mistaken for CPT inhibitors. On the basis of these results, while these compounds will not be discussed further in this document, the question is raised as to whether other compounds, particularly the aminocarnitine derivatives discussed in the following paragraphs, would also inhibit the CACT transporter and what implications this would have on their pharmacology. As no data are reported in the literature or known to us at the present time, this will remain a matter of speculation.

Following the same rationale, including consideration of transition state analogues, the phosphate derivative **12** (derived from the natural (*R*)-carnitine isomer) was reported by Sandoz (later Novartis) as a novel, reversible inhibitor of CPT1A,[111] where the putative tetrahedral ester oxyanion of the transition state was replaced by a tetrahedral phosphonate anion. Selectivity for CPT1A was to be achieved by selective targeting to the liver rather than intrinsic isoform selectivity, since the difference in inhibition of FAO in liver mitochondria and heart mitochondria was only 2- to 4-fold. However, no pharmacokinetic data on tissue distribution were reported. The (*R*)-isoform **12** was considerably more active than the (*S*)-isoform **13**. The phosphate analogue showed a similar activity, whereas the phosphinate and the phosphonamidate were substantially less active. The mode of inhibition of **12** was, as expected, reversible and competitive with respect to palmitoyl-CoA (K_i = 3.6 μM), and a chronic high-dose study was run to demonstrate that the compound does not induce cardiac hypertrophy. For this compound the well documented shift along the dose axis between FAO inhibition (i.e., KB lowering) and fasting glucose lowering was again demonstrated.[112]

Aminocarnitine and Its Derivatives. The antidiabetic properties of racemic and (*R*)-aminocarnitine or emeriamine **14** and its derivatization to nonhydrolizable acyl derivatives were reported in parallel by Jenkins and Griffith[113−115] as well as by researchers at Takeda Chemical Industries, which patented this class of compounds in 1984.[116−118] According to the reports by the Takeda group, emeriamine was the deacetylated derivative of a fungal metabolite, emericedin **15**, or acetylaminocarnitine, from *Emericella quadrilineata*, which demonstrated a weak FAO inhibitory effect in isolated rat liver mitochondria. (*R*)-Aminocarnitine **14** was a stronger inhibitor in vitro in isolated mitochondria (IC_{50} = 3.2 μM).[118] The long-chain acyl derivatives of aminocarnitine, particularly palmitoylaminocarnitine **16** and myristoylaminocarnitine, are considerably more potent inhibitors of FAO than aminocarnitine (IC_{50} = 0.050 μM for **16**).[114,117] Acetylaminocarnitine **15**, on the other hand, is a more potent inhibitor of carnitine acetyltransferase (CAT) than carnitine palmitoyltransferase, binding to the enzyme with a 13-fold higher affinity than the substrate acetylcarnitine.[114]

On the basis of the same legacy, substitution of the amide bond of palmitoylaminocarnitine with other types of linker, like ureido

Scheme 2. Structures of Representative Carnitine Analogues and Derivatives with the Most Common Use Names or Codes by Which They Appear in the Literature



or sulfonamide, led to a series of compounds described by scientists at Sigma Tau in the past decade. These compounds were demonstrated to be substantially more stable than palmitoylaminocarnitine to conditions in the stomach and gut. A series of interesting derivatives were described, for which activity was measured in rat liver and heart mitochondria to distinguish between CPT1A and CPT1B inhibitory activity.[119] Carbamate and carbonate derivatives incorporating the (*R*)-carnitine structure were essentially inactive, while the most potent compounds appeared to be the (*R*)-isomers of the C12-sulfonamide **17** and C11-sulfamide **18** of aminocarnitine. The sulfonamide **17** was demonstrated to be, as expected, a reversible competitive inhibitor with respect to palmitoyl-CoA ($K_i = 0.25\ \mu$M). The same authors expanded the SAR around these initial findings and identified the C14-ureido derivative of (*R*)-aminocarnitine **19** as the most interesting species. Although slightly less potent in in vitro assays, this entity showed an equally strong in vivo response in a chronic model of NIDD. The C11 derivative shows comparable efficacy, while shorter chain analogues were substantially less active.[120] Once again, competitive reversible inhibition with respect to palmitoyl-CoA was apparent ($K_i = 0.36\ \mu$M). To stress that the effect is not due to any putative detergent-like membrane-modifying property of these compounds, as it is sometimes postulated, it is noted that all pharmacological effects are stereospecific, with the (*S*)-isomer **20** showing no activity whatsoever either in vitro or in vivo. Disturbance of CPT2 activity by **19** was tested by checking its ability to inhibit the conversion of oleylcarnitine to oleyl-CoA in hepatocytes, and it was concluded that the compound does not affect CPT2. This is in contrast with our observation in isolated enzyme preparations (see Table 9). It was also demonstrated that **19** does not affect the PPARα receptor, CAT, and peroxisomal β-oxidation.[121] This series of compounds has been patented,[122] and compound **19** under the INN name teglicar entered clinical studies for T2D treatment in the early 2000s (see below).

An interesting aspect of all aminocarnitine derivatives is that although they have been reported as CPT1 inhibitors for a considerable part of their history, they are actually much stronger inhibitors of CPT2 in vitro and in vivo. The CPT2 isoform preference of (*R*)-aminocarnitine **14** was assessed in the early 1990s.[123] Huelsmann et al.[124] reported accumulation of long-chain acylcarnitine (LCAC) upon addition of aminocarnitine to perfusate in isolated rat hearts, which is an unequivocal marker of CPT2 inhibition. The IC_{50} for (*R*)-aminocarnitine (which we could confirm; see Table 9) was reported to be 0.80 μM at CPT2 and 19 μM at CPT1A.[125] Short-chain acylaminocarnitine derivatives are not strong inhibitors of CPT but rather of CAT. As demonstrated by the authors, longer chain acylaminocarnitines and other derivatives of aminocarnitines, including compound **19**, all have a preference for the CPT2 isoform in vitro (see Table 9).

Several other aminocarnitine derivatives were reported in the patent literature, mainly featuring extensive variations of the

Table 6. Summary of in Vitro and Ex Vivo Pharmacological Assays Performed with Carnitine Derivatives

compd	compd name	type of assay	biological matrix	assay conditions	efficacy	ref
12 and **13**	(*R*)- and (*S*)-SDZ-CPI-975	tissue and cellular	rat liver homogenates and hepatocytes form 18 h fasted rats	production of KB	For **12** IC_{50} of 36 μM in homogenates, 3.0 μM in isolated hepatocytes. The (*S*)-isomer **13** is inactive up to 100 μM in these conditions. NPAC **16** gives an IC_{50} of 1.3 and 1.2 μM in homogenates and hepatocytes, respectively.	111
14	(*R*)-aminocarnitine	subcellular	mitochondria isolated from 20 h fasted SD rats	radiometric determination of conversion of [1-^{14}C]palmitic acid, [1-^{14}C]palmitoylcarnitine, and [1-^{14}C]octanoic acid to $^{14}CO_2$	IC_{50} of 3.2 μM for oxidation of palmitate, ~12 μM for oxidation of palmitoylcarnitine, no inhibition of octanoate oxidation	118
			liver mitochondria isolated from 24 h fasted rats	radiochemical determination of enzyme activity	IC_{50} = 5.4 μM for palmitoylcarnitine oxidation. Extrapolated CPT2 IC_{50} of 0.8 μM.	125
		tissue	perfused rat hearts	addition of 52 μM aminocarnitine and/or 10 μM etomoxir to perfusion medium	Upon addition of aminocarnitine, massive increase in long-chain acylcarnitines in heart muscle. The effect can be reversed by addition of etomoxir. No effect on heart function and sarcolemmal integrity.	124
		ex vivo	liver mitochondria isolated from treated animals (18 mg/kg ip single dose) 24 h postdose	radiochemical determination of enzyme activity	Marked decrease in palmitate oxidation (−68%)	125
rac-**16**	palmitoyl-aminocarnitine	subcellular	detergent-lysed liver mitochondria from fasted rats	spectrophotometric assay with DTNB	$IC_{50} \approx 0.050$ μM.	114
17		subcellular	rat liver and heart mitochondria	radiometric determination of conversion of [^{14}C]palmitic acid to [^{14}C]palmitoylcarnitine	IC_{50} of 0.7 μM for liver and 3.4 μM for heart mitochondria. Under the same conditions, **12** has an IC_{50} of 17.4 μM for liver and 62 μM for heart mitochondria.	119
18		subcellular	rat liver and heart mitochondria	radiometric determination of conversion of [^{14}C]palmitic acid to [^{14}C]palmitoylcarnitine	IC_{50} of 0.8 μM for liver and 5.8 μM for heart mitochondria. Under the same conditions, **12** has an IC_{50} of 17.4 μM for liver and 62 μM for heart mitochondria.	119
19	ST1326	subcellular	rat liver and heart mitochondria	radiometric determination of conversion of [^{14}C]palmitic acid to [^{14}C]palmitoylcarnitine	IC_{50} of 1.1 μM for liver and 43.4 μM for heart mitochondria	120
23	ST2425	subcellular	rat liver and heart mitochondria	radiometric determination of conversion of [^{14}C]palmitic acid to [^{14}C]palmitoylcarnitine	IC_{50} of 0.13 μM (liver) and 5.44 μM (heart)	127
24	ST2452	subcellular	rat liver and heart mitochondria	radiometric determination of conversion of [^{14}C]palmitic acid to [^{14}C]palmitoylcarnitine	IC_{50} of 0.12 μM (liver) and 57.3 μM (heart)	127
25		cellular	several human cancer cell lines		GI_{50} between 0.1 and 1.8 μM	128
rac-**27**	HPC	subcellular	rat liver mitochondria	radiometric assay using [^{3}H]carnitine	IC_{50} of 15.5 and 47.5 μM for (−)- and (+)-HPC for CPT1A and IC_{50} of 6.7 and 38.5 μM for (−)- and (+)-HPC for CPT2	131

lipophilic side chain, which appears to tolerate a wide range of modifications. Compounds where the amide group of acylaminocarnitines is substituted by an heterocycle were claimed in 1999 and are exemplified by compounds **21** and **22** in Scheme 2.[126] Data are reported only for four compounds, which all show very modest inhibition at CPT1 with the DTNB method in isolated mitochondria. Giannessi et al. claimed a series of analogues of **19** bearing an aromatic group within the side chain,[127] in analogy to a former set of compounds by scientists at Sandoz.[12c] Of these, compound **23** (ST2425) appears to be the most interesting. The

Table 7. Summary of in Vivo Pharmacological Assays Performed with Carnitine Derivatives

compd	compd name	species	route	dose	observation	ref
12	SDZ-CPI-975	SD rats	po	2.7−11 mg/kg	Animals fasted overnight. Time course experiment. Significant reduction of KB levels, at all doses, maximal at 3 h postdose.	112
			po	0.44−88 mg/kg	3 h postdose. Dose response experiment. Glucose lowering starting at 22 mg/kg and −20% at 88 mg/kg. KB reduction already maximal at the lowest dose (−77% maximum).	112
			po	48 mg/kg	Muscle glucose utilization study: etomoxir as positive control (3.6 mg/kg). No effect on ex vivo muscle tissue from heart, soleus, or diaphragm.	112
		fat-fed, low dose STZ-treated rats	po	22−87.5 (mg/kg)/day for 11 days	6 h postdose after 6 h of fasting, dose dependent reduction of blood glucose down to normoglycemia.	111
		cynomolgus monkey	po	12.5 mg/kg	Animals fasted for 18 h prior to experiment. Time course experiment, 1−6 h postdose. Significant lowering of blood glucose only 6 h postdose. KB decrease also maximal 6 h postdose.	112
			po	0.125−12.5 mg/kg	Glucose lowering only at the highest dose. At the same dose, trend to increase in FFA. Significant reduction of insulin levels (−50%).	112
14	aminocarnitine	SD rats	po	0.3−30 mg/kg	Animals fasted 20 h. Rapid and strong decrease of KB already at 1 mg/kg, reduction of FBG at higher dose. No effect in fed animals.	118
		Wistar rats	ip	16 mg/kg	In fasted animals. Massive increase in plasma lipids: TG (+684%), FFA (+244%), VLDL (+1034%), LDL (+88%). Slight decrease in glucose (−16%). Significant increases in tissue TG and LCAC in heart, liver, and muscle. Decrease in spontaneous movement and hypothermia.	125
		STZ mice	ip and po	0.3 mmol/kg ip and 1 mg/kg po	Decrease in plasma glucose level starting 4 h postdose. Normoglycemia reached at 8 h postdose. Reduction of KB levels after 15 h of fasting and in the fed state.	114
rac-**16**	palmitoylamino-carnitine	mice	ip	0.1 mmol/kg	46% inhibition of the oxidation of [^{14}C]palmitate to $^{14}CO_2$. No effect on octanoate oxidation. Prevention of ketosis upon 24 h of fasting. Reversal of established ketosis. Signifant elevation of liver, kidney, and plasma TG.	114
		STZ mice	po	1 mmol/kg	Reduction of blood glucose by 45% at 6−12 h postdose	114
17		db/db mice	po	100 (mg/kg)/day for 30 days	Significant reduction of serum glucose levels (−28%), no effect on body weight, insulin, ALT, TG, and cholesterol. No cardiac or liver hypertrophy.	119
		rats	po	dose response	In 24 h fasted animals, KB (BHB) reduction 6 h postdose, ED_{50} = 20 mg/kg	119
19	ST1326	db/db mice	po	50 mg/kg b.i.d. for 45 days	Reduction of serum glucose (−25%), although no normoglycemia reached. Reduction of serum fructosamine (−30%, same level as lean mice). Reduction of liver glycogen (−25%). Reduction of water consumption (−30%) and polyuria. Elevation of liver TG (+38%) and circulating FFA (+20%). No effect on body weight, heart weight, TG content, plasma TG, and insulin.	120
			po	20 or 50 mg/kg b.i.d. for 45 days	Compound administered in postabsorption conditions. Eight hours after last treatment, no effect on blood glucose at 20 mg/kg b.i.d., −39% glucose reduction at 50 mg/kg b.i.d.	120

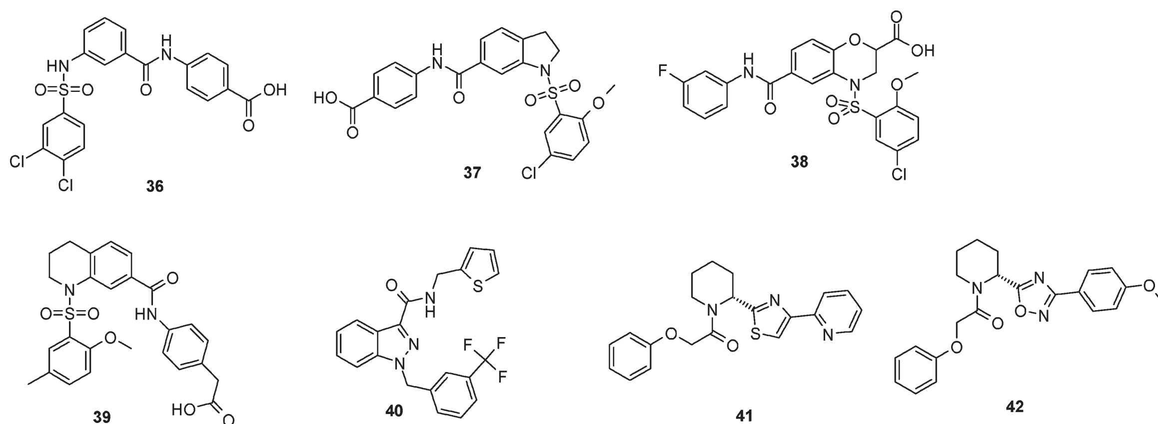
Table 7. Continued

compd	compd name	species	route	dose	observation	ref
			po	30 mg/kg with or without metformin (200 mg/kg) b.i.d. for 16 days, in postabsorptive conditions	No effect of **19** or metformin alone 6 h after the last dose. In the combination group, −25% glucose reduction.	189
			po	30 mg/kg with or without metformin (200 mg/kg) b.i.d. for 37 days, in postabsorptive conditions	OGTT in fasting conditions and 15 h after the last dose. Significant reduction of glucose AUC only in the combination group.	189
		ob/ob mice	po	30 mg/kg with or without metformin (200 mg/kg) b.i.d. for 11 days, in feeding conditions	No effect of **19** or metformin alone 15 h after the last dose. In the combination group, −27% glucose reduction.	189
			po	30 mg/kg with or without metformin (200 mg/kg) b.i.d. for 18 days, in postabsorptive conditions	No effect of **19** or metformin alone 8 h after the last dose. In the combination group, −30% glucose reduction.	189
		C57BL/6J mice on high fat diet	po	30 mg/kg with or without metformin (200 mg/kg) b.i.d. for 15 days, in postabsorptive conditions	No effect of **19** or metformin alone 6 h after the last dose. In the combination group, −27% glucose reduction, equal to lean group.	189
			po	30 mg/kg with or without metformin (200 mg/kg) b.i.d. for 35 days, in postabsorptive conditions	OGTT in postabsorptive conditions and 6 h after the last dose. Significant reduction of glucose AUC only in the combination group.	189
		rats	po	dose response	In 24 h fasted animals, strong dose-dependent reduction of BHB up to 12 h postdose. ED_{50} = 14.5 mg/kg	120
		SD rats	icv	one or two single doses (5−25 pmol)	Decrease in food intake and body weight on days 1−3 postdose. No effect of the inactive isomer **20**. Increase in GIR in insulin clamp studies due to significant decrease in glucose production (−45%).	219a
		cynomolgus monkeys	po	dose response	Inhibition of ketogenesis in fasted monkeys, ED_{50} = 0.5 mg/kg	121
		human	po	150 and 450 mg/day for 15 days	Well tolerated. Significant reduction of FBG was achieved at 450 mg, as well as a significant reduction of fasting insulin (−12%) and HOMA index (−25%).	190
23	ST2425	C57BL/6J mice	icv	250 pmol (0.113 μg)/day for 4 days	Reduction of food intake (−25%) and reduction of body weight (−7%)	127
		db/db mice	po	30 (mg/kg)/day for 12 days	Reduction of glucose levels (−41%). Under the same conditions, **19** at 80 (mg/kg)/day showed a −26% reduction.	127
		rats	po	dose response	Inhibition of ketogenesis in 17 h fasted animals, ED_{50} = 3.7 mg/kg, faster onset of action compared to **19**	127
		SD rats	intranasal	320 μg/day for 3 days	Significant reduction of food intake starting from day 2	127a
24	ST2452	db/db mice	po	30 (mg/kg)/day for 12 days	Reduction of glucose levels (−30%)	127

compound is more potent than **19** in vitro and in vivo in models of ketogenesis and diabetes. Interestingly, substitution of the trimethylammonium group with an equally charged triphenylphosphonium yields compound **24**, which retains similar activity in vitro and in vivo. Inventors at the University Health Network claimed over 100 compounds, partly overlapping with previous claims by Sigma Tau, of which **25** and **26** are representatives.[128] Several of the claimed examples were reported to have IC_{50} below 1 μM at CPT1A using an HPLC method for detection and quantification of palmitoylcarnitine in intact mitochondria from *Saccharmyces cerevisiae* expressing human CPT1A. The compounds were assessed in human cancer cell lines for their ability to inhibit proliferation (see below, therapeutic potential section), with compounds **25** and **26** exhibiting submicromolar GI_{50} in a variety of lines.

From the point of view of molecular properties, compounds **23**−**26** are permanently charged, highly amphiphilic molecules, which are expected to be endowed with detergent-like characteristics, namely, high solubility in water medium with a tendency to form micelles (however, compounds **23** and **24** are reported to have much higher cmc concentrations than **19** (0.79 and 0.55 mM vs 29 μM) due to the presence of the polar oxygens within the chain). Oral administration appears well tolerated, and the pharmacological effects are not due to any unspecific effects on membranes. However, ip administration has been shown to be highly toxic in rodents.[114] The permanent charge raises

Scheme 3. Structures of Representative CPT Inhibitors Identified by HTS Followed by Expansion and Optimization of the Hits



doubts on their permeation ability. We have generated a set of ADME data for **19** that show the compound to be highly soluble but poorly permeable and to have a very slow metabolism. The PK profile in rodents is consistent with poor bioavailability (2−4%), very high protein binding, and almost no clearance, leading to accumulation upon repeated dosing. The compound is distributed preferentially to the liver. These observations translated to a similar PK in humans (see section on therapeutic potential).[190]

Other Carnitine-Based Structures. Other types of structures derived from carnitine have been reported in the literature. Some attention has been dedicated to the cyclic structure represented by **27** (usually denominated hemipalmitoylcarnitinium or HPC), derived from the reaction of the methyl ester of (*R*)-norcarnitine with 1-bromo-2-heptadecanone.[129] The compound was intended to be a prototype of a bisubstrate transition state analogue and was found to be a relatively potent competitive inhibitor of purified CPT2 ($K_i \approx 0.16$ μM),[130,131] although it also inhibited the forward reaction in both heart and liver mitochondria. The competitive inhibitory constants vs carnitine were found to be 2.8 μM in heart and 4.2 μM in liver mitochondria.

Other cyclic carnitine derivatives have been reported but mostly show very weak inhibitory activity. The dehydrated HPC analogues were described as CPT1 and CPT2 inhibitors, but only single point data at 500 μM are reported.[132] These compounds also inhibit microsomal CPTs. Cyclooctane hybrids between HPC and **12** were described (**29** and **30**), but these are very weak CPT inhibitors ($IC_{50} > 100$ μM), while they are more potent inhibitors of PKC.[133] Other weak cyclic carnitine analogues like **31** ($IC_{50} = 19$ μM for FAO inhibition in hepatocytes) and **32** (IC_{50} in the millimolar range) were described.[12c] A recent patent[134] claimed cyclic carnitine analogues of general structure **33** for the treatment of cancer (see below, therapeutic potential section). The two isomeric structures **34** and **35** were described as having $IC_{50} < 1$ μM at CPT1A using an HPLC method for detection and quantification of palmitoylcarnitine, using intact mitochondria from *Saccharmyces cerevisiae* expressing human CPT1A. The growth inhibitory activity of all compounds of the invention against multiple human tumor cell lines was only modest.

CPT Inhibitors from HTS. There is no technical issue associated with the implementation of in vitro assessment of CPT inhibition to high throughput screening, particularly for the spectrophotometric method employing CoASH sensing with DTNB. To our knowledge, however, there is no reported HTS performed on any of the CPT isoforms in the literature. The authors have performed two distinct HTS experiments of the in-house compound library with the three separated CPT isoforms, identifying several chemotypes of potent CPT inhibitors with varying specificity. Some representative optimized structures are reported in Scheme 3 and Table 8. Details about the SAR and the in vivo pharmacology of these compounds will be reported in due course.

These compounds are the only existing CPT1A inhibitors for which selectivity over CPT1B and CPT2 is demonstrated to an extent that allows exclusion of any contribution of heart and muscle to the pharmacological action. In particular the phenoxyacetamide series systematically show over 10000-fold selectivity for CPT1A over the other two isoforms.

Miscellaneous CPT Inhibitors. Four well-known antianginal compounds that do not show any hemodynamic effects are reported to effect at least part of their well-described pharmacological action via CPT1 inhibition, namely, **43** (trimetazidine), **44** (perhexiline), **45** (amiodarone), and **46** (oxfenicine) (Scheme 4).[142]

Compound **43** is an antianginal drug commercialized by Laboratoires Servier under the trade name Vastarel, at a dose of 35 mg/b.i.d. It is effective in patients with heart failure of different etiologies and well tolerated.[143] Trimetazidine (**43**) increases coronary flow reserve, limits rapid swings in blood pressure without any significant variations in heart rate, significantly decreases the frequency of angina attacks, and improves left ventricular function in diabetic patients with coronary heart disease. Compound **43** inhibits fatty acid oxidation in the heart and in the blood vessel, promoting glucose oxidation in the heart and limiting the drop of ATP production during ischemia. Its inhibition of CPT1B in rat myocardium, however, is very weak (IC_{50} = 1.3 mM).[144] It has been postulated that **43** exerts its effect by inhibiting another enzyme of the FAO cascade, 3-ketoacyl-CoA-thiolase (LC 3-KAT),[145] although the precise mechanism of action is still a matter of discussion.[142] The compound has also been used effectively in a model of fasting hyperglycemia[146] in rats, but here again the low dose used (1 mg/kg) suggests that the MOA is not through CPT1. Another compound analogue to **43**, compound **47** (S15176), has been reported to be a more potent CPT1 inhibitor (IC_{50} = 16.8 μM in heart and 51 μM in liver mitochondria, confirmed in ex vivo studies).[147]

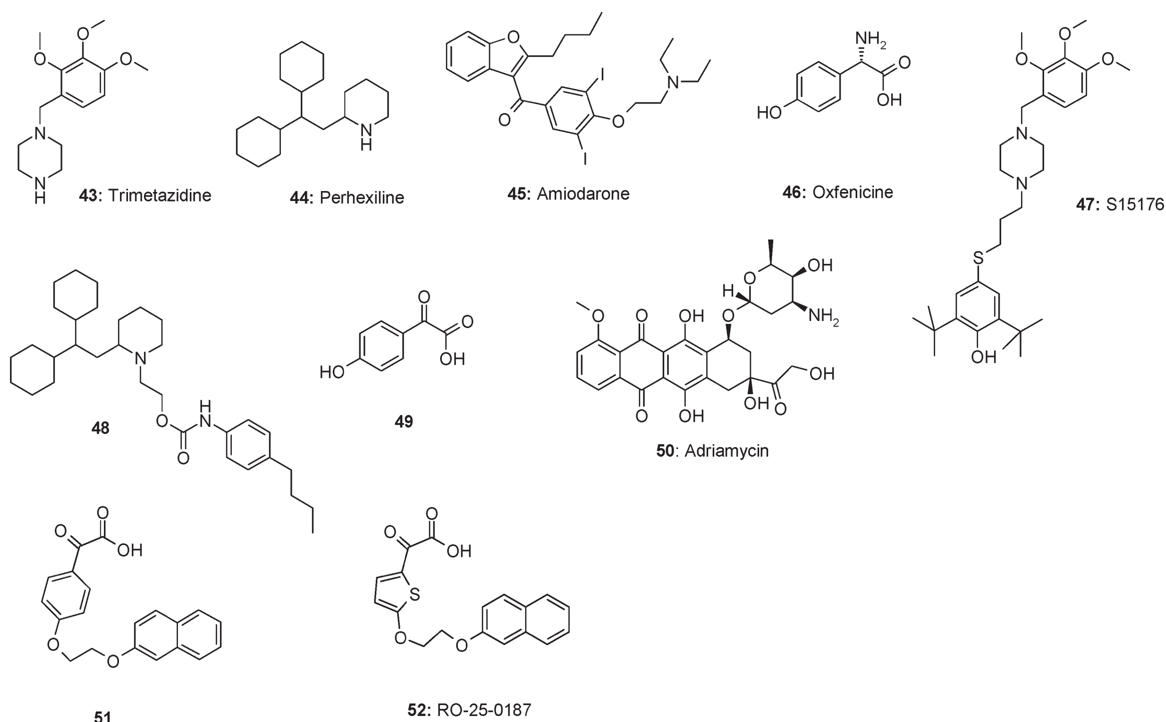
Perhexiline (**44**), frequently prescribed as an antianginal agent in the 1970s, is currently used almost exclusively in Australia.[148]

Table 8. Enzymatic and Cellular Activity of New CPT Inhibitors Identified by Optimization of HTS Hits

compd	CPT1A (h/r) IC_{50} (μM)[a]	CPT1B (h) IC_{50} (μM)[a]	CPT2 (h/r) IC_{50} (μM)[a]	KB (rat hepatocytes) IC_{50} (μM)[b]	ref
36	0.19/−	6.4	−/28.5	4.5	135
37	0.021/0.055	>100	0.74/3.2	1.09	136
38	0.016/0.030	3.4	>100/>100	1.34	137
39	0.16/0.18	3.5	2.8/16.7	0.026	138
40	0.026/0.15	>100	−/>100	2.95	139
41	0.020/0.13	>100	4.8/30.7	0.41	140
42	0.065	>100	−/>100	0.21	141

[a] See ref 177. [b] See ref 178.

Scheme 4. Structures of Miscellaneous Compounds Reported to be CPT Inhibitors



There is evidence that it shifts the metabolism of the myocardium from FAO to glucose utilization, and it is believed that this occurs through inhibition of CPT1 (IC_{50} of 77 and 148 μM in rat cardiac and liver mitochondria, from radiometric method using ^{3}H-carnitine)[149] and CPT2 (IC_{50} = 79 μM), resulting in increased lactate and glucose utilization.[150,151] Its use has been restricted and in most countries banned because of its liver and peripheral neurotoxicity and limited therapeutic window. In patients with CYP2D6 deficiency (~10% of the caucasian population), the drug is poorly metabolized and reaches dangerous concentrations, leading to the majority of reports of adverse events. The hepatotoxic and neurotoxic effects have been attributed to CPT1 inhibition.[152] However, given that toxic effects are observed when the plasma drug concentration exceeds 0.6 mg/L (~2 μM), this appears unlikely, as it is questionable that the metabolic shift in the heart is due solely to CPT inhibition. Perhexiline reduces ATP content and cell viability in isolated rat hepatocytes already at 25 μM and inhibits oxidation of short, medium, and long-chain fatty acids as well as oxidative phosphorylation in vitro and in vivo in mice,[153] an effect that is not seen with much more potent CPT1 inhibitors. It has been reported, however, that the concentration of the drug in rat heart is at least 20-fold that in plasma 3 h postdose.[149] Still, in consideration of the fact that perhexiline actually increases acetyl-CoA levels in perfused rat heart (2 μM in the perfusate), doubts remain about the effective contribution of CPT inhibition to its pharmacology.[151] On the basis of the structure of perhexiline, scientists at Sigma Tau have claimed a number of derivatives, exemplified by **48**, for which they claim CPT1A activity and which are intended for use in the central nervous system. Unfortunately, no in vitro or in vivo data are reported for these compounds in the patent.[154] Compound **48** was chosen randomly and assessed in our panel of assays, but had IC_{50} > 10 μM at both rat CPT1A and rat CPT2.

CPT1A inhibitory activity has been reported also for the antiarrythimic drug amiodarone (**45**) (IC_{50} = 140−228 μM).[149] However, although for **45** 90-fold accumulation in dog heart[155] has also been reported, both its pharmacological and numerous adverse effects seem unrelated to CPT activity. The mechanism of action of **45** is still unknown.

The hydroxyphenylglycine oxfenicine (**46**) was intended as a cardiac drug that stimulates glucose over FA metabolism in the

Table 9. Spectrophotometric in Vitro IC_{50}[177] of selected CPT modulators in recombinant *Pichia pastoris* Membrane Preparations and Effect on β-Oxidation Rate (FAO) and KB Production in Isolated Human and Rat Hepatocytes[178,179]

compd	name	CPT1A IC_{50} (μM) human/rat	CPT1B IC_{50} (μM) human	CPT2 IC_{50} (μM) human/rat	KB IC_{50} (μM) rat	FAO IC_{50} (μM) human/rat
rac-**1b**	TDGA[a]	–/–	–/–	–/–	0.31	<0.05/0.04
rac-**2b**	POCA[a]	–/–	–/–	–/–	59.7	10.4/95.9
6a	etomoxir[a]	–/–	–/–	–/–	2.76	0.29/0.34
7	DET[a]	–/–	–/–	–/–	5.6	1.8/4.4
14	aminocarnitine	76.4/27.3	>100	3.9/5.1	0.97	–/–
16	palmitoylaminocarnitine	2.67/–	21.7	0.14	0.11	2.8/0.27
19	ST1326	1.05/1.45	5.4	0.16/0.38	1.1	4.0/0.98
23	ST2425	1.70/–	85.3	5.4/–	0.21	0.73/0.03
43	trimetazidine	>100/–	>100	>100/–	47.1	20.5/>100
44	perhexiline	>100/–	>100	72.8/–	14.8	22.4/21.4
45	amiodarone	>100/–	>100	>100/–	16.6	28.5/>100
46	oxfenicine	>100/–	>100	>100/–	>100	>100/>100
49	oxfenicine metabolite	>100/–	60.3	>100/–	25.8	33.3/>100
52	RO-25-0187	2.4/–	0.16	4.7/>100	1.5	25.5/>100
55	C75[a]	–/–	–/–	–/–	36.5	21.9/>100
56	C75*[a]	–/–	–/–	–/–	50.1	77.0/>100

[a] These derivatives cannot be assessed in isolated enzyme assays, as they cannot be converted to the -CoA derivatives. Moreover, thiol-reactive agents like DTNB are not adequate to measure malonyl-CoA sensitivity of CPT1A or CPT1B.[180] This constitutes a limitation of the assay. For this reason, the -CoA derivatives of oxirane carboxylic acids and **55** could not be assessed in this assay.[181]

cardiac muscle[156] and has beneficial effects in models of heart failure.[157] The active species is actually its metabolite 4-hydroxyphenylglyoxylate (HPG) **49**, which is produced directly in the heart by the resident transaminases. Besides this type of metabolic tissue targeting, **49** also shows intrinsic specificity for CPT1B (IC_{50} of 11 and 510 μM for heart and liver mitochondria).[158] Compound **46** affects FAO in heart muscle in a chain-length-dependent manner, which appears consistent with its effects on CPT1B.[159] The compound causes heart hypertrophy and other adverse findings in some animal models, which were speculated to be a consequence of CPT inhibition.[160] Another cardiotoxic compound for which CPT activity was reported is the antineoplastic agent adriamycin (**50**). Also in this case, the CPT activity is extremely weak (IC_{50} = 250 μM at CPT1 and IC_{50} > 100 μM at CPT2 in isolated rat mitochondria)[161] and the compound inhibits oxidation of fatty acids independent of chain length, pointing to alternative target(s).[162] More potent CPT1 inhibitors analogous to **50** have been reported that do not show cardiac toxicity.[163]

A series of malonic acid analogues were designed by scientists at Roche with the intent to inhibit selectively the CPT1B isoform, which is more sensitive than CPT1A to malonyl-CoA allosteric control.[164] These compounds resemble the active metabolite of **46**, having an α-keto acid as a recurring structural element. Examples of such compounds are **51** and **52** (RO-25-0187). These compounds are reported to bind not to the catalytic site but to the second, allosteric binding site of malonyl-CoA, which does not require the CoA group for affinity.[30] The compounds are fairly potent and selective inhibitors of CPT1B, with IC_{50} in the low submicromolar range, although they also inhibit CPT1A with variable potency (see Table 9).

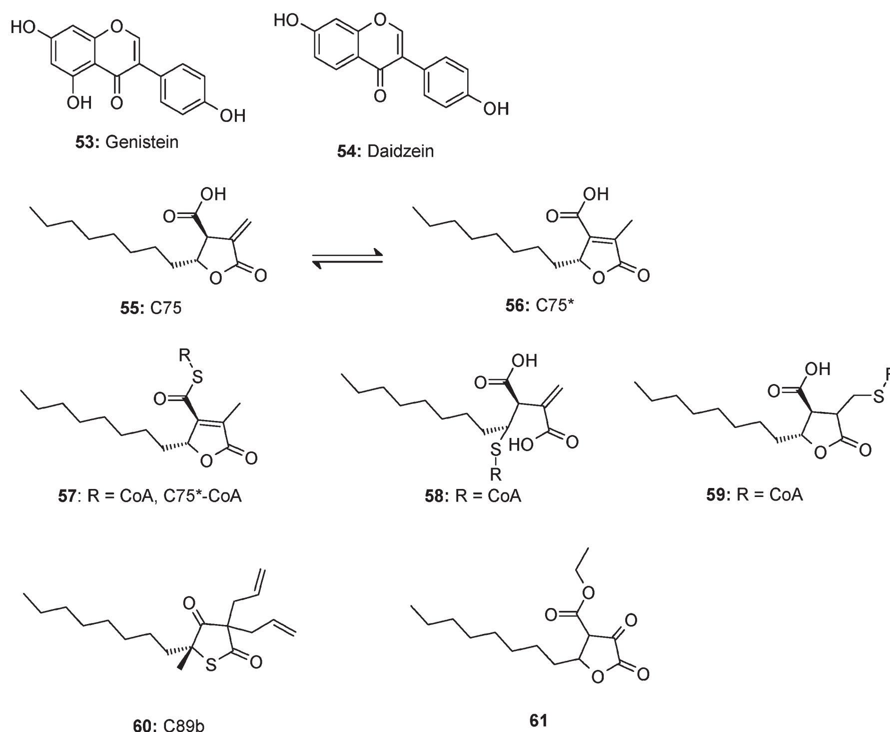
■ CPT ACTIVATORS

As CPT1 activity is subject to a number of controlling elements, it is difficult to distinguish between direct activation and effects on the mitochondrial membrane or the cytosol composition that indirectly influence CPT1 activity. CPT expression is stimulated by metformin and AMPK[165,166] and is one of the gene targets of PPARα,[167] and its expression is therefore stimulated by all PPARα activators. Given that CPT1 has an allosteric inhibition mechanism, direct activation by a neutral binder to the allosteric site is conceivable.

The soy isoflavones genistein **53** and daidzein **54** (Scheme 5) were reported to increase CPT1A mRNA and enzyme activity in hepatocytes cell lines treated with 10 μM concentration of these compounds, particularly in the presence of (*R*)-carnitine.[168] Another report, however, described decreased CPT1 activity in db/db mice treated with these compounds, in parallel with a whole range of beneficial metabolic effects.[169]

The most prominent compound reported to be a direct activator of CPT is compound **55** (C75). This compound is a slow-binding competitive inhibitor of fatty acid synthase (FAS) identified by scientists at the The Johns Hopkins University.[170] On handling this compound, we soon realized that the structure described in the literature is not chemically stable and tends to interconvert to the tautomer **56** (C75*) in solution. The conversion kinetics, measured by NMR in DMSO solution containing variable amounts of deuterated water,[171] shows that the conversion is facilitated by small amounts of water and is probably very rapid in physiological medium as well as in test solutions handled under ambient atmosphere. Moreover, the thioester derivatives of type **57** (e.g., with R = *N*-acetylcysteamine), having increased acidity at the α-carbon, exist only in the tautomer form. While it appears that a CoA conjugate of **55** forms in vivo in several tissues and it may be a determinant of the pharmacological activity of **55**, it is not totally obvious what is the molecular species involved. If formation of the CoA adduct is mediated in vivo by acyl-CoA synthase, then this is likely to be a thioester (**57**), existing only in the more stable tautomer form in physiological conditions. Hegardt et al.[70] proposed that the CoA derivative of **55** corresponds to the product of ring-opening of the lactone (**58**) and later revised the structure of

Scheme 5. Structures of Compounds Reported to be CPT Activators



this entity as the product of Michael addition of the thiol to the exocyclic double bond (**59**),[172] as can be expected from chemical reactivity consideration. We could also confirm that this structure is formed by simply mixing thiols and **55** in physiological conditions in the absence of any enzymatic catalysis. The CoA derivative produced outside physiological systems and corresponding to structure **59** is a CPT1A (IC_{50} = 0.24 μM) and CPT1B (IC_{50} = 0.36 μM) reversible inhibitor in preparations of the isolated enzymes expressed in *Pichia pastoris*. Compound **55** itself, however, increases the activity of CPT1 in these liver and muscle mitochondrial yeast extracts by 20−30% at 10 μM.[70] In another study, no activation effect of **55** was visible in a similar assay system.[172] Despite the assumption that **55** (an activator of CPT) is converted to the CoA conjugate (an inhibitor of CPT) in physiological systems, the net effect appears to be that of an activator of FAO in vivo and in cells, as it increases energy expenditure in whole animal calorimetry in mice (15 mg/kg, ip), which was reversed by etomoxir cotreatment, and enhances palmitate oxidation by 750% and CPT1 activity (radiometric measurement with [^{14}C]carnitine) by 250% at 80 μM in primary rat hepatocytes. As this effect was also visible in the presence of inhibitory concentrations of malonyl-CoA (and **55**, being a FAS inhibitor, increases physiological concentrations of malonyl-CoA considerably), it was concluded that **55** prevents binding of malonyl-CoA to its inhibitory site and is possibly a neutral binder at the malonyl-CoA site.[173] However, by use of CPT1B expressed in *Pichia pastoris* as well as intact hepatocytes, it was shown that **55** does not displace malonyl-CoA, although it does overcome inactivation by malonyl-CoA and by the malonyl-CoA mimic **52**.[174] Compound **55** has a central hypophagic effect, but whether this comes from CPT1 activation[175] or CPT1 inhibition[172] in the hypothalamus or is the effect of the other pharmacology of **55** is a matter of controversy (see below).

Activators of CPT1 devoid of FAS activity (**60** and **61**) were later described, of which **60** (C89b) is the most characterized example.[176] Compound **60** increased CPT1 activity, measured by increase in the levels of radiolabeled palmitoylcarnitine in MCF-7 breast cancer cells and increased oxidation of fatty acids by 177% at 31 μM (estimated EC_{50}: 3 μM, 140% control).[175] Compound **60**, incidentally, had less potent but longer lasting hypophagic effect than **55** when admistered icv in mice.

■ COMPARATIVE DATA OF REPRESENTATIVE CPT MODULATORS

We have generated a set of data, including isoform specificity, for representative historical CPT modulators under the same assay conditions. Although the assay system available to us (DTNB method[177]) has limitations with regard to CoA derivatives and oxirane carboxylic acids, we believe it can help comparative assessment of legacy compounds. Data on inhibition of KB production and FAO in rat and human hepatocytes were also generated (Table 9).

The data in Table 9 actually demonstrate that many well described inhibitors are unspecific. The case of aminocarnitine derivatives is particularly striking, as most of these compounds that are claimed as selective CPT1A inhibitors are actually stronger inhibitors of CPT2 in vitro. The putative liver specificity of this intervention is therefore not given, and the effects of systemic inhibition of FAO might be expected if the CPT2 inhibition translates in the in vivo setting.

■ THERAPEUTIC POTENTIAL OF CPT MODULATORS

CPT1A Inhibitors. Hepatic glucose production, which is a sum of gluconeogenesis and glycogenolysis, is the main contributor to

fasting hyperglycemia in type 2 diabetes.[182] The relationship between fatty acid oxidation in the liver and gluconeogenesis has been long established and extensively reviewed.[183]

In liver, FAO appears essential for maximal rates of gluconeogenesis and it has long been observed that inhibitors of FAO decrease gluconeogenesis. Extensive preclinical evidence supports an antigluconeogenic effects of liver specific CPT inhibitors, with both in vitro systems and animal models (see Tables 4 and 5). Hepatocytes from fed rats treated with oleic acid show an increase in gluconeogenesis, which can be reversed by addition of the nonselective CPT1A/CPT1B inhibitors **1**. In animal models, direct evidence of gluconeogenesis inhibition by **1** was obtained by studying the conversion of labeled pyruvate to labeled glucose.[48]

The mechanism(s) responsible for the inhibition of hepatic gluconeogenesis by liver specific CPT1 inhibitors can be multiple.[48] Inhibition of FAO could limit ATP production, which is necessary to fuel the gluconeogenesis cascade. The ATP content in hepatocytes in the presence of the gluconeogenesis substrates pyruvate or lactate is, however, not affected by **1**.[48] Inhibition of FAO also limits NADH generation, which would affect gluconeogenesis from all substrates entering upstream of glyceraldehyde 3-phosphate dehydrogenase. However, ethanol, which acts as an extramitochondrial electron donor, was not able to reverse inhibition of gluconeogenesis by compounds **1** from pyruvate, lactate, or alanine.[48] This suggests that the decrease of reducing equivalents brought about by **1** (as indicated by reduction of the cytoplasmic lactate/pyruvate ratio)[48] is not the underlying mechanism for the reduction of gluconeogenesis from the three-carbon substrates. The most solid link between reduction of FAO and reduction of gluconeogenesis appears to be pyruvate carboxylase. This enzyme is activated by high intramitochondrial levels of acetyl-CoA. Compounds **1** deplete acetyl-CoA concomitant with accumulation of long-chain fatty acyl-CoA, as demonstrated in fasted rat hepatocytes by Williamson.[184] This will lower pyruvate carboxylase activity. Indeed compounds **1** failed to inhibit glucose production from proline, which enters gluconeogenesis at the oxalacetate level.[48]

It appears well substantiated that inhibition of hepatic fatty acid oxidation via inhibition of CPT1A will bring about a reduction of gluconeogenesis. The quantitative link, however, appears more fragile.

Almost all CPT1 inhibitors ever described have been shown to affect glucose levels. As none of these compounds is selective for the liver specific CPT1 isoform and may affect either CPT1B or CPT2 at the systemic level, these results do not aid the quantification of the direct relationship between inhibition of CPT1A, inhibition of gluconeogenesis, and reduction of glucose levels. Low doses of **1a** (<2.5 mg/kg), which inhibit only CPT1A but not CPT1B, were not able to affect the blood glucose levels of 48 h fasted rats, although CPT1A was strongly inhibited and ketone bodies were lowered as much as 90%.[75] McGarry and Foster also observed a disconnection between the reduction of FFA oxidation and gluconeogenesis in experiments with refeeding of fasted rats.[185] Compound **1a** was not able to interfere with hormonal control of gluconeogenesis[186] or with the increase of gluconeogenesis associated with insulin-induced hypoglycemia.[187] All detailed studies report the order of events: CPT1 inhibition *before* KB reduction *before* glucose reduction on both the dose and time axis as discussed previously and confirmed by us (a graphical representation that distills and condenses knowledge from many different experiments reported in the literature or performed by us is shown in Figure 6), which could reflect the necessity to reach plasma levels of inhibitor sufficient to affect peripheral glucose utilization through either CPT1B or CPT2 inhibition. The selective CPT1A inhibitors identified by the authors are able to affect glucose levels in rodent models of T2D only at doses that are several-fold over maximum enzyme inhibition and maximum plasma KB reduction. These observations cast some doubts that CPT1A inhibition alone might be sufficient to significantly affect blood glucose levels in diabetic organisms unless hyperglycemia is due to highly overregulated gluconeogenesis. Among the factors that may concur in buffering the effect of CPT1A inhibition alone on gluconeogenesis and glucose levels are (a) the multiple and rapidly reacting regulation of CPT1A expression and deinhibition in response to the physiological status, (b) the competitive nature of many active-site binding CPT1A inhibitors (with the exception of the irreversible inhibitors of the oxirane carboxylic acid class), (c) the possibility of compensatory mechanisms feeding the intramitochondrial acetyl-CoA pool independently of mitochondrial FAO, for example, peroxisomal FAO.[188] In general, while the rationale seems well established, some skepticism on the quantitative effects that could be expected on diabetic hyperglycemia from pure CPT1A inhibitors remains. Nonselective inhibitors, which also affect peripheral glucose utilization, may have a more robust efficacy in this context. Nevertheless, in a patient population with highly overregulated gluconeogenesis as the major contributor to hyperglycemia and in which glycemic control is not achieved by standard therapy, a CPT1A inhibitor could be a valuable alternative option. A patent by Sigma Tau scientists suggests that a synergistic effect could be expected by combination of a CPT1A inhibitor with established antidiabetic agents. In combination with subpharmacological doses of metformin, **19**, a nonselective CPT1A/CPT2 inhibitor with only modest activity at CPT1B demonstrated a synergistic effect in lowering blood glucose in fed and postabsorptive conditions in ob/ob, db/db, and DIO mice models after subchronic treatment, while the two components singly administered at the chosen doses were not able to elicit an effect. The combination also had a synergistic effect on glucose AUC in an OGTT performed after several days of treatment in both db/db and DIO mice. The implicit suggestion is that CPT1A inhibitors at low doses could be a valuable add-on therapy to improve glycemic control in treated T2D patients.[189]

Two clinical studies were initiated in NIDD with compounds that were assumed not to affect peripheral glucose utilization. After characterization in rodent and primate models, which confirmed an effect on FAO and fasting glucose,[112] albeit at relatively high doses, compound **12** entered phase I studies sponsored by Sandoz/Novartis. No data were ever reported on this study, which was very rapidly terminated because of mitochondrial toxicity observed in preclinical models.[12c] More information is available on the Sigma Tau sponsored study involving the well-characterized CPT1A/CPT2 inhibitor **19** or teglicar. This compound is still reported to be in active development, although progress appears to be very slow. Recently, data on the phase I results were disclosed.[190] In a double blind randomized placebo-controlled study, 40 NIDD subjects on standardized diet (age 35−75 years, FBG 7−14 mmol/L, HbA1c 6.5−9%, diet alone or single medication) were treated with 150 and 450 mg/day of **19** for 15 days. The compound was well tolerated with no significant changes in vital signs, physical examination, ECG, hematology, and clinical chemistry. Some

moderate gastrointestinal discomfort appeared to be the only dose-limiting event. The PK of the compound showed accumulation behavior, with a $T_{1/2}$ of 25 h. A significant reduction of FBG was achieved at 450 mg, as well as a significant reduction of fasting insulin (−12%) and HOMA index (−25%). The conclusion by the sponsor was that the 450 mg once daily dose appeared well-tolerated and adequately efficacious, although the antidiabetic effects at this dose were not very impressive.

On the other hand, all liver-acting inhibitors of CPT, be it through CPT1A or CPT2, are extremely potent antiketogenic compounds in all animal models used, independent of whether ketosis is generated by prolonged fasting or is a consequence of simulation of T1D, like in STZ, alloxan, or depancreatized animals (see Tables 5 and 7). The antiketogenic effect has immediate onset at very low doses and is often the first pharmacologic biomarker observed. A potential use of CPT inhibitors in the treatment of diabetes ketoacidosis (DKA) was already suggested by Tutwiler et al. in 1978.[46] Although the therapeutic protocol for emergency intervention in ketoacidosis is well established,[191] this condition is still associated with a relative high mortality rate (∼5% in the Western world). The intervention protocol is complex and risky, requiring experienced personnel, and relapse is frequently observed once insulin therapy is discontinued. It is conceivable, although not yet demonstrated, that an antiketogenic compound in the form of a CPT1A or CPT2 inhibitor could be a valuable addition to the treatment protocol for DKA, possibly limiting the incidence of complications, simplifying the protocol, reducing recovery time, and/or reducing mortality.

CPT1B Inhibitors. The idea that T2D results from insulin resistance in muscle (causing decreased glucose uptake) and liver (causing increased gluconeogenesis), combined with declining β cell function, is now widely accepted. For several decades, it was believed that the biochemical basis of hyperglycemia in T2D could be explained by the Randle cycle or glucose−fatty acid cycle, which describes the reciprocal effects of glucose oxidation and FAO in controlling each other's rate.[192] By this principle, increased fatty acid oxidation causes a commensurate decrease in glucose oxidation, leading to decreased glucose uptake and hyperglycemia. Increased availability and use of fatty acids by muscle were moreover regarded as a key component of the etiology of insulin resistance. Randle was also the first to propose that a pure inhibitor of FAO should improve glucose utilization.

Although this view is an oversimplification,[193] the concept of the Randle cycle was behind the search for inhibitors of muscle fatty acid oxidation for decades and fits the in vivo effects observed for muscle specific CPT inhibitors.

CPT1B is the regulation point for fatty acid oxidation in the muscle. This isoform is allosterically inhibited by the metabolite malonyl-CoA. This guarantees that in conditions of high glucose oxidation, where the concentration of malonyl-CoA is high, the rate of FFA uptake into the mitochondria and the corresponding oxidative process is maintained to a minimum. On the other hand, FAO produces acetyl-CoA, which is an inhibitor of pyruvate dehydrogenase, and citrate, an inhibitor of phosphofructokinase. Both enzymes are part of the glucose catabolism; therefore, in conditions of high FAO (for example, during fasting and exercise), glucose oxidation is negatively regulated. This complex network of reciprocal regulation guarantees that no "idle cycles" take place and allows the continuous adaptative substrate selection that tissues have to make between glucose and fatty acid oxidation. On this basis lies the therapeutic potential of CPT1B inhibitors in both diabetes and cardiac diseases.

A number of experiments confirmed that compounds like **1**, **2**, and **6** (dual CPT1A/CPT1B inhibitors) exert their effect on glucose reduction at least partly if not mainly via enhancement of glucose uptake by the muscle, especially after chronic treatment. In animals treated with **1a** (25 mg/kg, po, Table 5) 2 h before sacrifice, an increase in glucose oxidation rate (approximate doubling) in hemidiaphragms secondary to impairement of FAO was observed, supporting the relationship between FAO and glucose utilization in noncardiac muscle.[47] Kinetic studies with tracers have confirmed that the hypoglycemic effect of **6** in fasted animals is mainly due to an increase in peripheral glucose utilization. In fasted pigs infused with **6b** and [U-^{14}C,3-^{3}H]-glucose, the metabolic clearance rate increased by 126% from 5.0 mL/kg min in the control group to 11.3 mL/kg min in the treated group, while no effects were seen on glucose turnover rates and recycling of glucose carbon.[194] In rats, an acute dose had little effect on glucose utilization, while chronic treatment increased glucose turnover and recycling of glucose carbon by 40%.[195] On the other hand, Collier et al. clearly demonstrated a strong decrease in hepatic glucose production after an overnight fast in STZ rats treated chronically with **6a**.[196]

The mechanism of the hypoglycemic effect of these nonselective inhibitors in humans is also controversial. While **1a** was assessed in humans only in a single case study of massive hyperglycemia due to the development of insulin autoantibodies (where it was demonstrated to affect gluconeogenesis),[90] **6a** was extensively studied in human diabetic patients. Reduction of fasting blood glucose and ketone bodies was achieved uniformly under all study conditions to a more or less discrete degree. While in some cases clamp studies revealed an increase in glucose clearance rates due to increase in peripheral glucose utilization (e.g., under high insulin conditions),[98] in other instances the reduction of circulating glucose could be attributed solely to a reduction in gluconeogenesis, which was attributed to a competition effect of the elevated plasma FFA at the level of extrahepatic tissues.[95] Hubiger et al. could measure an increase in oxidative utilization of glucose concomitant with FAO reduction in NIDD patients treated with **6a** for 3 days via isotope dilution mass spectrometry and indirect calorimetry but could not measure any alteration of the glucose infusion rate under euglycaemic clamp conditions.[97] In another study, no effect on glucose utilization could be determined via indirect calorimetry or euglycaemic clamp after 3 days of treatment with **6a**, although a clear effect on liver gluconeogenesis could be assessed.[95] In one study, an activation of the pyruvate dehydrogenase complex (PDHC) by a single dose of **6b** was documented in heart, WAT, and liver tissue in either lean or obese mice but not in quadriceps muscle,[197] while other authors reported activation of PDHC in quadriceps muscle after chronic treatment with subpharmacological doses of **6b** in rats.[195] The activity of this enzyme, a possible mediator of the glucose/fatty acid cycle, is controlled by the intracellular level of acetyl-CoA and therefore responds to the rate of FAO. These findings suggest that while glucose utilization may be influenced by treatment with **6** in tissues other than liver, the muscle may respond to the altered rates of FAO only after longer treatment periods.

FFAs are the primary substrate for energy production in the heart. Oxidation of free fatty acids provides considerably more ATP than oxidation of glucose but requires more oxygen per mole of ATP produced than glucose oxidation (P/O ratio for palmitate is 2.83; P/O ratio for glucose is 3.17). During hypoxia associated with an ischemic episode, therefore, a switch from

lipid consumption to glucose consumption could be beneficial for maintaining heart function. Moreover, a decrease in FFA metabolism in the heart via CPT1B inhibition could lead to a decrease of FFA metabolites which are considered detrimental to cardiac function and contribute to the cardiovascular risk in dislipidemia, particularly long-chain acylcarnitines (LCAC)[198] (although **6a** was shown to improve cardiac function independently of the levels of LCACs).[85,88]

Oxfenicine **46** reduced infarct size by 25% in dogs subjected to LAD ligation and reduced levels of the cardiotoxic acylcarnitines[199] and improved ventricular function in a model of low-flow ischemia in the swine.[200] Glucose consumption was not measured in these studies, and the positive effect was attributed to the reduction of toxic metabolites. Renstrom et al. measured a 2-fold increase in glucose oxidation in the swine model with oxfenicine treatment and preservation of heart function during ischemia but no effect of treatment during the reperfusion phase.[201] In humans with CAD, iv administration of oxfenicine significantly increased the time to onset of angina during rapid atrial pacing.[202] It is noted, however, that oxfenicine **46** is an extremely weak CPT1B inhibitor in vitro, and even compared with the high doses employed in the studies, it appears highly questionable that the effects seen with this compound are due to CPT1B inhibition.

More convincing are the results produced with oxirane carboxylic acids. In rat perfused hearts,[79] **1b** prevented the negative effect of oleate perfusion under ischemic conditions. In vivo in swine, however, **1b** did not improve mechanical function during the ischemic period.[82] Treatment with **2b** and **1b** in isolated perfused rat hearts subjected to global ischemia caused a significant decrease in ATP levels during ischemia, associated with delayed functional recovery. However, LVDP and ATP production was improved over control 20 min after reperfusion.[80] In another study, **1b** was shown to improve cardiac function after subtotal ischemia only in diabetic hearts.[81] More consistent with the theory are the results produced by Lopaschuck et al. with **6**. Treatment with **6a** improved functional recovery after global ischemia in rat hearts independently of its effect on lipid metabolites and increased glucose oxidation rates significantly.[85,87] The same effect was observed in isolated perfused hearts from STZ-treated rats.[88] In diabetic rats treated with **6a** (18 mg/kg/day ip) for 6 days, heart function was improved, despite an increase in heart (and liver) lipid content.[86] In a rat model of hypertrophied pressure-overloaded heart (ascending aorta constriction), chronic low-dose treatment with **6a** prevented myocardial and ventricular changes associated with the transition from compensated hypertrophied to failing hypertrophied heart.[89,203] These findings indicate that oxirane carboxylic acids may be a valuable therapeutic option for the treatment of cardiac failure.

These results were pivotal to the initiation of human studies with **6a** in congestive heart failure. An initial uncontrolled study in 10 patients suffering from heart failure showed that 3 months of treatment (80 mg/day) improved stroke volume and cardiac output during exercise, as well as increased left ventricular ejection fraction also at rest. Acute inotropic or vasodilatory effects were excluded.[204]

Although these results appear as a confirmation of the metabolic switch hypothesis, the positive effects observed with oxirane carboxylic acids in the failing heart muscle are probably not due solely to inhibition of FAO in this organ.[205] Compounds **1**, **2**, and **6** all bring about an alteration in the expression of dysregulated genes which are important for hearth function, like SERCA2, Ca^{2+}-ATPase and α-myosin heavy chain.[206] PPARα activation by these compounds may contribute to restoration of the reduced PPARα activity in hypertrophied myocardium. Metabolic remodeling, however, is considered a key element of the action of oxirane carboxylic acids on the heart, and an excessive utilization of FFAs by the heart has been suggested to be one of the elements contributing to heart disease.[207] This was reflected in the acronym chosen for the largest clinical study of etomoxir in congestive heart failure, the ERGO (etomoxir for the recovery of glucose oxidation) study, which was sponsored by the German−American company MediGene.[208] Etomoxir **6a** was planned to be tested for 6 months at 2 doses (40 and 80 mg/day) in 350 patients with ischemic heart disease, recurrent angina, and in about 80% of the sample, a history of previous myocardial infarction. Only a fraction of the patients recruited (17%) had NIDD. Unfortunately, the study was terminated because of liver transaminase elevation observed in 4 patients (of 226 treated). At the time, only 21 patients on placebo, 16 on the low dose, and 14 on the high dose group had completed the study, a number too small to achieve statistical significance on the end-points chosen, although a trend toward a dose-dependent increase in exercise time was apparent. Only modest and transient effects on liver enzymes had been reported in previous studies, while in this case the effect appeared to be strong and persistent, indicating liver cell toxicity. There was also a marked difference in the patient population compared to the previous, nonplacebo controlled study. Thus, evaluation of the potential of CPT inhibition in cardiac disease remains unattained.

It is noted that a recent report confutes the hypothesis that FAO inhibition in the heart by **6** impairs FFA uptake or utilization by rat cardiomyocytes and points to a different MOA for **6** in improving cardiac function.[209]

The potential combination of antidiabetic and cardioprotective effects suggests CPT1B inhibitors as the optimal intervention in advanced NIDD, complicated by cardiovascular liabilities. Oxirane carboxylic acids, with their additional effects on circulating lipids and expression of key cardiac genes, as well as renal protective effects observed in rodent models of diabetes,[44,210] represent an extremely interesting molecular entity whose medicinal chemistry and therapeutic potential has not been exhaustively explored.

CPT2 Inhibitors. Because of its ubiquitous expression, systemic inhibition of CPT2 could potentially recapitulate or synergise the effects of CPT1A and CPT1B inhibitors.

The sometimes considerable inhibition of CPT2 by aminocarnitine analogues suggests that enhancement of glucose utilization by the muscle and other tissues cannot be excluded a priori in their antidiabetic properties. It is a fact, however, that the preferential inhibition of CPT2 by **19** and its analogues in isolated enzyme preparations, where the mitochondrial membrane has been disrupted, does not translate in coherent observations in more complex systems consisting of either cells or intact mitochondria. If this was the case, then inhibition of palmitoyl-CoA oxidation should be visible in muscle and in liver mitochondrial preparation (which is apparently not the case; see ref 127a). This discrepancy can be attributed to the poor permeability of **19** and analogous carnitine derivatives through the phospholipid bilayer. As CPT2 is located on the inside of the mitochondrial membrane, these compounds may not reach their preferred target whenever the membrane is intact, and FAO

inhibition observed in such systems is due only to CPT1A inhibition.

It is also conceivable that when CPT1A is particularly deinhibited or overexpressed (low malonyl-CoA concentration, high glucagon/insulin ratio, high carnitine concentration, fasted conditions, diabetes), then CPT2 becomes the gate keeper of acyl-CoA entry into the FAO cycle. McGarry et al. observed similar rates of oxidation of acylcarnitine in fed and fasted animals, while the fasted animals had much higher rates of oxidation of oleate, pointing to a sort of "rate-limiting" effect of the conversion of acylcarnitines to acyl-CoA that is not regulated by feeding status. Indeed, while a lot of information is available on the physiological regulation of CPT1, very little is known about the dynamic range of CPT2 activity. It remains to be elucidated whether in vivo effects of **19** and analogues are due to CPT1A, CPT2, or combined inhibition. As many of the aminocarnitine-derived CPT2 inhibitors show some degree of liver targeting, tissue specific inhibition of CPT2 could be an additional therapeutic option.

CPT2 inhibition is associated with increase in plasma and tissue long-chain acylcarnitine (LCAC) levels, which is the most distinctive characteristic of intervention at this enzyme and is a good marker of CPT2 inhibition.[125] This bears its own consequences in terms of safety profile (see below).

Nonselective CPT Inhibitors and Other Aspects of Therapeutic Potential. As almost all described CPT inhibitors are nonselective inhibitors, the preclinical and clinical effects described for these compounds are likely a combination of different pharmacological effects. It appears well demonstrated that the antidiabetic effect of **19** and **6**, which are nonselective CPT1A/CPT1B inhibitors, is due to a combination of reduction of hepatic gluconeogenesis and increased glucose utilization by the muscle. Alteration of glucose metabolism and insulin resistance often coexists with ischemic heart disease and contributes to the progression of this condition. The fact that inhibition of FAO at the systemic and hepatic level may relieve dysfunctional glucose metabolism and have a beneficial effect on the ischemic heart in a synergistic manner offers an attractive option for patients where both conditions exist.[211]

The oxirane carboxylic acid **1a** was studied in animal models of type 1 diabetes, like depancreatized dogs.[46,78] In this model, **1a** showed the best effect in dogs having low but detectable insulin levels and having hyperglycemia closer in severity to that of juvenile diabetes. These results and a small study with **1a** in T1D patients[212] suggest a potential therapeutic value of CPT1 inhibition in T1D and MODY.

CPT1 mRNA was reported to be increased in the skin of psoriatic donors, which led to the suggestion that CPT1 inhibitors might be beneficial in psoriasis, where inhibition of FAO may curb cell proliferation. Compounds **6** were reported to have effects comparable to those of betamethasone in a xenograft model of human psoriasis.[213]

The antiproliferative effect of CPT inhibitors in certain cell types has led to the hypothesis that this intervention may be beneficial for specific types of tumors. It was found that CPT1C and CPT1A are up-regulated in specific types of solid tumors and are determinant to cell survival and proliferation in hypoxic conditions, like those experienced locally by rapidly dividing tumor cells. Depletion of CPT1C in mouse embryonic stem cells led to reduced cell proliferation and sensitization to apoptosis signals, glucose deprivation, and hypoxia, which are often observed in tumors. CPT1A depletion in tumor cells had a similar effect, leading to increased cell death. The effect of CPT1A inhibitors of the acylaminocarnitine class on tumor cell lines appears promising.[128,134]

Leukemia cells cultured on a feeder layer of mesenchymal stromal cells were shown to have an uncoupled phenotype, with increased FAO rates. Indeed, pharmacological inhibition of FAO with **6a** in leukemia cells decreased proliferation and sensitized the cells to the apoptotic effect of the Bcl-2 inhibitor ABT-737 and the MDM-2 antagonist Nutlin 3a. In such combinations, **6a** showed therapeutic benefit in a murine leukemia model and decreased the number of quiescent leukemia progenitor cells in peripheral blood or bone marrow of acute myeloid leukemia patients. These results tend to support the evaluation of FAO inhibitors for the treatment of leukemia and support the link between fatty acid metabolism and leukemia cell proliferation and apoptosis.[214]

A further effect often observed with oxirane carboxylic acids is the reduction of total cholesterol as well as of triglycerides and FFAs upon chronic treatment in several animal models[215] and in humans,[95] which is perpendicular to the increase in circulating FFAs and TGs observed in acute settings and with other types of CPT inhibitors. This led to the suggestion that these compounds could be especially beneficial in dislipidemic diabetic patients. This effect is almost certainly due to the documented off-target effects of oxirane carboxylic acids (see above) and is not secondary to CPT inhibition, although it is potentially a very interesting element of the pharmacological profile of this class of compounds.

A further aspect of the pharmacological profile of oxirane carboxylic acids that could be of interest for therapeutic intervention is the apparent renal protective effect, which may also be consequent to PPAR-α activation.[210]

Besides these more obvious metabolic effects, it should not be forgotten that long-chain acyl-CoA esters are active modulators of several cellular processes, like gene expression, membrane fusion, gene transcription, membrane composition, and the activation of PKCs. In particular, acyl-CoA esters activate ATP-sensitive K^+ channels. Moreover, they are substrates for the synthesis of complex lipids and ceramide. Any CPT modulator has the potential to alter the intracellular lipid content and composition, with as yet unforeseen effects (positive or negative) on a number of interdependent systems.

Central Modulation of CPT. The central nervous system (CNS) is known to be a key regulator of whole body homeostasis. It is also well-known that a major CNS area, the hypothalamus, is devoted to the homeostatic control of thermoregulation and energy expenditure. Several lines of evidence raise the possibility that certain hypothalamic nuclei sense the availability of peripheral nutrients, including glucose and fatty acids, and hormones/cytokines such as insulin, adiponectin, ghrelin, glucagon-like peptide 1, and leptin. Indeed, it has been shown that an increase of nonesterified fatty acids and/or glucose hypothalamic levels activates efferent neural circuits that in turn suppress endogenous (hepatic) glucose production and food intake.[216] It has also been shown that malonyl-CoA may act as a sort of neurotransmitter in the hypothalamus, where it seems to be involved in central regulation of energy homeostasis and food intake.[217,218]

Two CPT1 isoforms, CPT1A and CPT1C, are expressed in the brain and are believed to be involved in central control of energy homeostasis and feeding behavior. The hypothesis that CPT1C is the target of malonyl-CoA in this context has been

advanced:[19] CPT1C binds malonyl-CoA, but it is not clear whether any catalytic function is relevant to its physiological role. Lane et al. demonstrated that CPT1C KO mice exhibit reduced food intake and body weight but also decreased rate of FAO and higher susceptibility to high-fat-diet-induced obesity, which suggests that CPT1C is involved in the central regulation of energy homeostasis through its sensitivity to malonyl-CoA.[19]

On the other hand, genetic and pharmacological interventions have allowed the recognition, among others, of two biochemical sensors associated with the neuronal metabolism of lipid: the malonyl-CoA/CPT1A axis.[217,219] Indeed, the icv infusion of a sequence-specific ribozyme against CPT1A or of compound **19** in the third cerebral ventricle or in the mediobasal hypothalamus (MBH), which resulted in a significant decrease of hypothalamic CPT1 activity, led to a marked reduction of hepatic glucose production, as measured in in vivo experiments involving pancreatic clamps and food intake in overfed rats and mice.[219] Moreover, the orexigenic action of ghrelin, a well-known gastrointestinal hormone that controls food intake and appetite, is mediated via the activation of the AMPK-malonyl-CoA-CPT1 axis of hypothalamic neurons.[220] Current data demonstrate that pharmacological or genetic manipulations of the AMPK-malonyl-CoA-CPT1 axis in the VMH induce marked changes in the expression of neuropeptides in the ARC.[221]

On another note, scientists at The Johns Hopkins University support the notion that CPT1 inhibition in the hypothalamus actually increases food intake and that CPT1 activators are hypophagic. According to these authors, icv admistration of **6a** increases food intake and body weight due to rebound hyperphagia after an initial weight loss.[175] Others report exactly the opposite effect with **6a**, although with a shorter follow-up of the experiment, leaving an open question on the effect of kinetics.[172] As for the putative CPT activator **55**, differential effects on food intake and body weight are reported for lean vs obese rodents (with the effects in lean rodents being somewhat similar to those seen with **6a**), the solution of the conundrum could lie in the metabolic status of the animal model used.[222]

It has also been suggested that the rate of whole body fat oxidation in itself is a cue for a metabolic satiety signal.[223] According to this hypothesis, decrease in FAO rate should increase hunger feeling and food intake, quite at an angle with the legacy of animal studies where no effect of peripheral CPT inhibition on body weight was ever observed. Two placebo controlled studies in humans who either were naturally consuming a high fat diet or were fed a high fat diet as part of the study could not establish a clear correlation between reduction of FAO by **6a** and hunger and satiety feeling.[91,92] A recent study with **6a** in subjects deprived of time cues could also not establish a correlation between FAO inhibition and satiety variables.[224]

In summary, the real effects of central (and peripheral) CPT modulation on control of hunger and satiety and liver glucose production remain a matter of controversy and observations are probably complicated by fuzzy pharmacology.

CPT Activators. A strong rationale exists for the potential utility of FAO activation in the treatment of obesity. Reduced levels of lipid oxidation are associated and are predictive of obesity in humans and rodents.[225] Activation of lipid catabolism, especially in the muscle, which is the most dominant tissue in the regulation of energy homeostasis, appears to be an attractive option for the treatment of obesity. Activation of CPT1B, possibly via reduction of the strict malonyl-CoA allosteric control over this enzyme, is a possible target in this context. Reduced CPT1B activity has been demonstrated in obese patients.[226] In rodents, genetic or pharmacologic reduction of malonyl-CoA levels results in increased lipid oxidation, decreased adipose mass, and protection from diet-induced obesity and diabetes.[227] Clinton et al. reported that overexpression of CPT1A in muscle of high-fat diet insulin-resistance rats improved muscular insulin resistance.[228] However, genetic or pharmacologic increase of muscle malonyl-CoA significantly improves insulin sentivity in DIO mice.[229]

Considering only direct intervention at the level of CPT1 and not any indirect activation via gene regulation or alteration of the operating milieu of the enzyme, pharmacological data in this direction have been collected for the CPT1 activators **55** and **60**. Compound **55** increased fatty acid oxidation in rodent adipocytes and hepatocytes, as well as in human breast cancer cells, by increasing CPT1 activity.[173] Animals treated subchronically (10 days) with 20 mg/kg ip every 48 h showed a 20−22% reduction of body weight over the course of the experiment coupled with an acute reduction of food intake after each dose. DIO mice treated with **55** (15 mg/kg ip, single dose) had a 32.9% increase in energy production and lost more weight than pair-fed animals (4.4% vs 2%) at 20 h postdose, indicating that the weight loss is an effect of increased energy expenditure and not of reduced food intake.[173] Compound **55** acts centrally to reduce food intake through a mode of action in which the role of CPT1 activation or inhibition is not clear (see above).[230] The compound also reduced adipose mass and fatty liver in obese rodents.[222] **55**, being a FAS inhibitor, increases the levels of malonyl-CoA. Its CPT1-activating effects, therefore, are taking place in the presence of increased level of an allosteric inhibitor of the same enzyme. To avoid this contrasting pharmacology, the analogues **60** and **61**, devoid of FAS activity, were identified.[176b] Compound **60** stimulated FAO by 150% at 28 μM and stimulated CPT1 activity by 150% at 60 μM in hepatocytes, and **61** had very similar pharmacology. Compound **60** at 100 mg/kg po induced a greater and more persistent weight loss than pair-fed animals (on top of reduction of food intake) in a DIO mice model.[176b]

Figure 7 recapitulates in a schematic way the therapeutic venues potential accessible with CPT modulators.

■ SAFETY ASPECTS OF CPT MODULATION

As described above, the CPT system is a central element of energy homeostasis. It is expected that exogenous inhibition or activation of any of the enzymes of this family has the potential to induce metabolic derangements and to affect the energetic balance of several tissues. This, together with the implicit alteration of fatty acid metabolism and disposal, may well lead to toxic events that limit the potential therapeutic application of CPT modulators. From another perspective, while it might be safe to intervene in the rate of FAO in diseased subjects, this same intervention might be detrimental in individuals where this process is in equilibrium, a typical conundrum in the design of clinical development plans involving healthy volunteers. The time axis is another dimension to be considered in the assessment of the safety issues potentially associated with CPT modulation, as toxicity might manifest itself only upon chronic treatment with CPT modulators. An impressive amount of data and considerations on the safety (or lack thereof) of CPT modulation have been reported in the literature. This topic merits, therefore, some extended discussion.

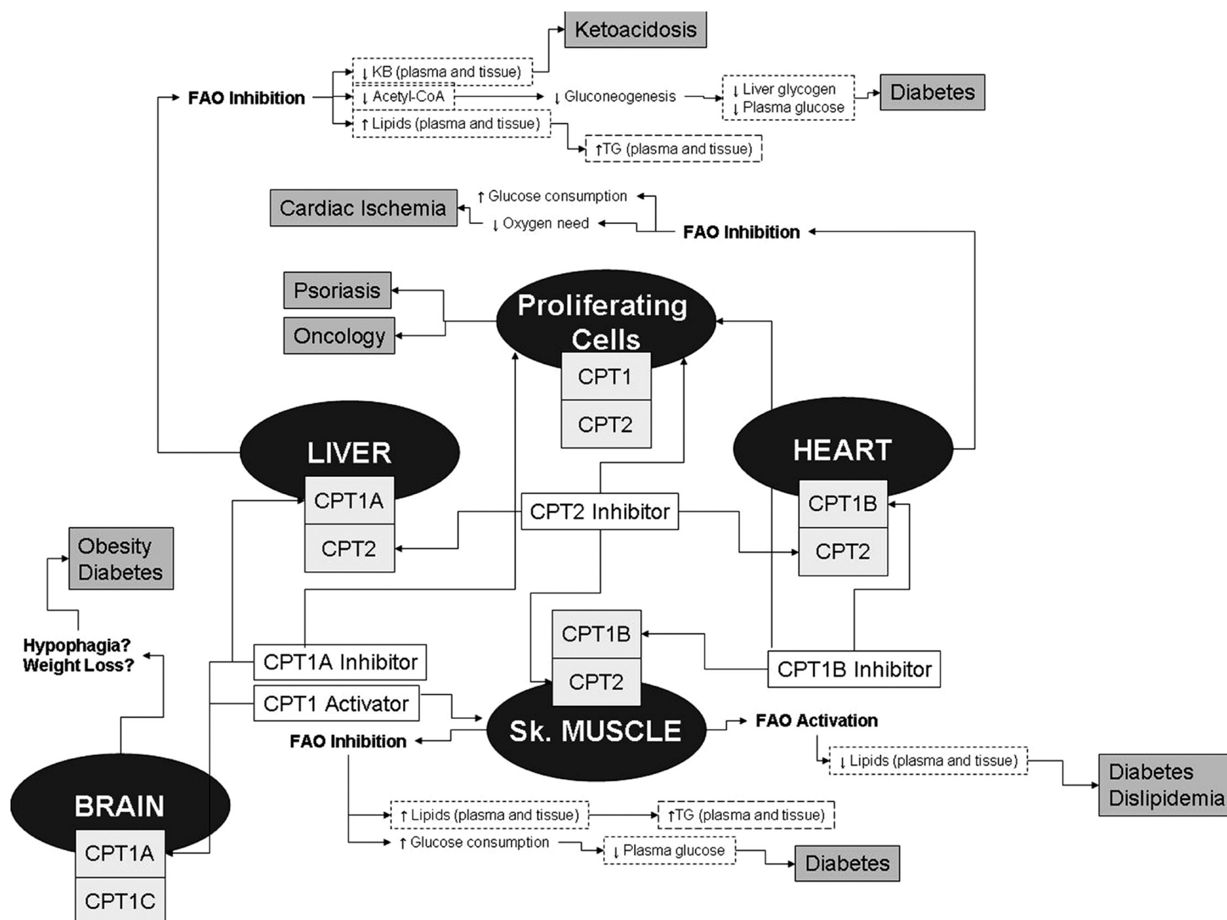


Figure 7. Summary representation of a classical view on potential therapeutic applications of CPT modulators. Hashed frames circle components that can serve as biomarkers of intervention in the CPT system. Nonselective inhibitors could show a combination of effects. Potential side effects are not indicated and are discussed extensively in the safety section.

The isoform specificity of the putative inhibitor or activator plays a great role in the assessment of the potential therapeutic index. For example, it is a general perception that selective CPT1A inhibition, not affecting muscle FAO, would be a safer approach to reduction of hepatic β-oxidation and consequent reduction of HGP. However, all preclinical and clinical data relating to reduction of fasting glycemia in T2D models or patients via inhibition of CPTs were generated with unselective inhibitors. Intrinsic liver specificity ("targeting") of the molecular entities discussed above is also an element to be considered in the evaluation of target-related safety. Carnitine-based compounds, for example, show a substantial higher concentration in the liver compared to other tissues in rodents (unpublished data).

A further element of confusion in this matter is the potential off-target effects demonstrated by etomoxir **6**, one of the most profiled CPT1 inhibitors. This compound has been reported to be a PPARα agonist, and it is not clear whether this is a direct effect of the molecule[205] or is a downstream effect of CPT1 inhibition.[231] In the former case, this off-target activity could well contribute to some of the toxic effects seen with this compound.

Genetic Deficiencies. CPT1A deficiencies represent a rare metabolic disorder. Typical features of CPT1A deficiency include hepatomegaly, hypoketotic hypoglycemia, and disturbed liver function. There are no cases of CPT1B deficiencies reported, probably because of incompatibility with life. CPT2 deficiencies, on the contrary, are relatively common in the population.[232] Inborn CPT2 deficiency has three distinct clinical phenotypes: (i) a relatively common adult myopathic form, (ii) a severe infantile form, and (iii) a lethal neonatal form. The adult onset form of the disorder primarily involves the skeletal muscle and shows a stress-triggered phenotype characterized by voluntary muscle myalgias and rhabdomyolysis following prolonged exercise, fasting, high fat intake, viral illness, extremes in temperature, or drugs such as diazepam, ibuprofen, and valproic acid.[232b] The attacks last from a couple of hours to several weeks. Serum creatine kinase (CK) and transaminases are often increased during the attacks. Skeletal muscle structure is apparently normal in 50% of these patients, and type 1 muscle fibers are mostly affected. More than 60 disease-causing mutations have been reported.[233] These have a range of effects on the enzyme's activity, substrate specificity, regulatory interactions, and stability. Similarly, phenotypic manifestations are apparently resulting from these amino acid substitution mutations. CPT2 deficiency is diagnosed by determining enzyme activity in leukocytes and fibroblasts, which ranges from 5%- to 25% of control values. It appears that the residual FAO rate in fibroblasts correlates better with the severity of the phenotype.[234]

In the more severe CPT2 deficiencies in infants and neonates, mostly organs highly dependent on FAO (particularly in this

early age) are affected (liver, heart, skeletal muscle), in which up to 95% activity can be lost. The severe neonatal fatal form, in addition, is characterized by dysmorphogenesis mainly affecting brain and kidney. These severe cases of inborn almost complete and continuous loss of CPT2 might not be relevant for adult T2D patients under a therapeutic intervention.

On the basis of these data, however, it is assumed that full continuous enzyme inhibition of any of the three most well-described isoforms would lead to substantial toxicity. Less clear is the impact of intermittent or moderate (up to 80%) enzyme inhibition. In the case of CPT2 inhibitors, possible reasons of concern for inhibition appear to be the potential for rhabdomyolisis following stress situations or prolonged exercise and the putative effect on fetal development effect, as suggested by the severity of the neonatal isoform.

Cardiac Safety. The oxirane carboxylic acid **2b** administered as a food admix to rats (about 200 (mg/kg)/day) for 12 weeks caused enlargement of the heart muscle by 15% (as well as liver lipid accumulation and reduction of VLDL and HDL) with no functional or macroscopic structural changes.[235] Incidentally, no further significant safety-related findings were identified in this study, despite the high dose (compared to the hypoglycemic dose of 5−10 mg/kg in fasted rats) and the length of treatment. Also chronic administration of **1a** was found to produce left ventricular hypertrophy and a decrease in left-ventricular compliance.[236] High pharmacological dose (100 mg/kg) of **6a** induced left-ventricular hypertrophy in a 10-day study in rats.[237] Oxfenicine treatment also produced cardiac hypertrophy in rat and dog hearts.[238] Despite many hints in this direction in the review literature, it is not at all demonstrated that oxirane carboxylic acids or oxfenicine induce cardiac hypertrophy secondary to CPT1B inhibition. All oxirane carboxylic acids induce peroxisomal proliferation via PPARα and alter massively the gene expression pattern in the heart, and this might be at the origin of the cardiac hypertrophy effects in animals. The doses at which hypertrophy is observed are several-fold higher than the pharmacologically relevant doses. Aminocarnitine derivatives, many of which are potent CPT2 inhibitors and therefore potentially affect β-oxidation in the heart, do not cause cardiac hypertrophy,[239] which speaks against a mechanism related effect. On the other hand, chronic etomoxir treatment was shown to improve cardiac function in a number of models despite, or even because of, induction of mild left-ventricular hypertrophy.[89] It is important to note that diabetic animal models, such as db/db mice, exhibit increased fatty acid utilization and oxidation in the heart. It is therefore anticipated that T2D patients with comparable alterations in heart metabolism could profit from FAO inhibition, according to the metabolic switch hypothesis.

Treatment for 3 months with **6a** (80 mg/day) in patients suffering from heart failure did not induce any detectable cardiac hypertrophy,[204] nor was any such finding reported in the conclusions of the ERGO study.[208]

Published data on antiangina drugs that are reported to switch metabolism in the heart toward glucose utilization such as trimetazidine, perhexiline, amiodarone, and oxfenicine do not provide a clear-cut evidence linking CPT inhibition to cardiac safety issues, although this has been suggested multiple times. The compounds are only extremely weak CPT inhibitors, and their full target profile has never been satisfactorily elucidated.

A marked increase of long-chain acylcarnitines (LCACs), coupled with a sharp decrease of carnitine and acetylcarnitine, was observed by the authors and others[239,124] in several models upon acute selective inhibition of CPT2. LCACs have documented cardiac toxicity in several ex vivo and in vitro models and have been implicated in ischemic injury.[198] A quantitative extrapolation to the physiological situation following CPT2 inhibition is difficult because of the extremely high protein binding of these amphiphilic molecules and because of the limited dose-response studies available. Treatment of perfused rat hearts with the CPT2 inhibitor aminocarnitine brought about a massive increase in LCACs in the heart muscle, which were released in the perfusate. However, no impairement of cardiac function or loss of sarcolemmal integrity was observed. The authors concluded that increase in LCAC alone, without acidosis, does not impair cardiac function.[124] Increase in circulating LCAC is also seen in human CPT2 deficiency[240] without any documented sign of cardiotoxicity comparable to that observed in ex vivo models. The clinical significance of this factor alone is therefore unclear.

Increase in Tissue Lipid Levels and Effects on Insulin Resistance. The association between circulating and tissue lipids and declining insulin sensitivity is well documented.[241−243] Approaches aimed at reducing lipolysis and circulating FFAs are considered promising approaches to treat insulin resistance. While it appears logical to expect that inhibition of β-oxidation would lead to accumulation of unoxidized lipids in the tissues affected and possibly to an increase in circulating lipids, the evidence concerning the effect of CPT inhibition on insulin sensitivity is rather controversial.

Treatment of C57BL6/J mice with **1b** (30 mg/kg ip) was reported to induce massive microvesicular hepatic steatosis within 12 h.[244] Chronic treatment of rats with **6a** (0.01% food admix, 4 weeks) led to an increase of intramyocellular lipids, which was associated with a significant drop of insulin mediated glucose disposal rate, as measured by hyperinsulinemic-euglycemic clamp.[245] Kelly et al., on the other hand, reported a substantial accumulation of lipids in cardiac myocytes and liver upon treatment with **6a** (50 (mg/kg)/day, 5 days) only in PPARα −/− mice.[231] The CPT2 inhibitor aminocarnitine was reported to generate massive increase in circulating and tissue lipids in fasted rodents[125] but not in fed animals. Reports from adult CPT2-deficient patients showed that muscle lipid storage is found in 20% of patients.

In a clinical setting, treatment with **6a** resulted in a decrease of circulating triglycerides and LDL/HDL ratio (see Table 5), an effect that had been observed also with the analogue **2** in animal models, although concomitant with an increase in liver lipids.[235] The reduction of circulating lipids clearly appears to be due to off-target effects of the oxirane carboxylic acids, which inhibit both fatty acid and cholesterol synthesis via an unknown mechanism, although at higher doses than FAO inhibition.[101] The PPARα agonistic activity demonstrated by all oxirane carboxylic acids might be at the origin of these effects and might also explain the findings by Kelly et al., described above, although it should be kept in mind that PPARα −/− mice also have defective carnitine metabolism.[246] If, as it appears likely, this is an intrinsic property of the molecules and not a consequence of CPT1 inhibition, all in vivo effects of the oxirane carboxylic acids have to be interpreted in this light, as discussed above for the effect of oxirane carboxylic acids on cardiac and renal function, and this class of compounds cannot be compared head to head with other types of CPT inhibitors.

In general, it is assumed that pure CPT inhibition in tissue will cause a mechanism-related increase of tissue lipid content and eventually of circulating lipids.

Because of an increase in plasma FFA and liver and muscle TG accumulation, concerns can be raised about the worsening of insulin sensitivity. There are examples of TG accumulation and hepatic steatosis, which do not induce insulin resistance, such as overexpression of DGAT2,[247] or acute administration of the oxirane carboxylic acid **1a**,[248] and the hypothesis has been raised that fatty acids contribute to the etiology of insulin resistance because of their excessive oxidation, leading to mitochondrial overload.[249,250] Compound **6a** increased the IC_{50} for the inhibitory effect of oleic acid on insulin binding in hepatocytes by 3-fold, which was taken as indication that FAO is an important element of the inhibitory effect of fatty acids on insulin binding in isolated hepatocytes.[251] A recent report showed that increased FAO in endothelial cells causes overproduction of the reactive oxygen species superoxide, which activates proinflammatory signals leading to vascular damage and markedly decreased two antiatherogenic enzymes, prostacyclin synthase and eNOS. This effect could be prevented in obese Zucker (fa/fa) rats by inhibition of FA release from the adipose tissue with niacine or by inhibition of CPT1 by **6b**.[252] This study would point to a beneficial effect of FAO inhibition in the vascular endothelium regardless of the level of circulating lipids and supports the notion that excessive FAO contributes to the etiology of insulin resistance and that FAO inhibition would be beneficial in this context.

Chronic 45-day treatment of db/db mice with **19** did not worsen insulin sensitivity.[120] Human data with **19** demonstrate a reduction of circulating insulin in treated patients, which would at least support the notion that CPT inhibition does not worsen insulin sensitivity. Inhibition of CPT1A in β-cells was even associated with increased insulin secretion.[253]

In summary, the effect of CPT inhibition and of the concomitant increase in tissue and plasma lipids on insulin sensitivity is controversial. Theoretically, arguments for both a beneficial and a negative effect have been advanced. Animal and human data generated with oxirane carboxylic acids are not useful in this context, while data with aminocarnitines in animals and humans are not discouraging. There is no evidence that liver steatosis in rodents resulting from FAO inhibition is linked with inflammation and steatohepatitis (NASH).

Niacin prevented the acute plasma FFA and TG increase seen with **6a**, and a synergistic effect on glucose lowering was observed.[254] This finding points to the potential pharmacological value of a combination of FAO inhibitors and antilipolytic agents.

Other Safety Concerns. While notably lacking in more recent literature, older reports contain observation of severe hypoglycemia associated with some CPT inhibitors in animals. High doses of both **1a** and **6a** were associated with hypoglycemia and even coma and death when administered to fasting animals.[55,78] In fed conditions, glucose levels are generally only minimally affected. In long-term dose-range finding studies in rodents, acute toxicity of **6** and establishment of LD_{50} were based on hypoglycemic coma (other findings were mild increase in organ weight of liver, heart, and kidney without histopathologic changes).[96]

Interference with targets so tightly involved in mitochondrial function and involved in the control of β-oxidation may raise concerns of potential mitochondrial toxicity.[255] Mitochondrial lesions are associated with steatosis, inflammation, apoptosis, necrosis, and fibrosis, and there is a relation between pharmacologically induced liver injury and mitochondrial toxicity in some instances. The CPT inhibitors **6a** and **12** were assessed for mitochondrial safety in rat and human liver slices.[256] Both compounds provided evidence of changes in mitochondrial morphology in both human and rat liver tissue, associated with a decrease of ATP and GSH content as indexes of liver tissue disfunction at high concentrations (100−500 μM for **6a** and 250 μM for **12**). Compound **6a** also showed evidence of mitochondrial disfunction in rat in an ex vivo study (125 mg/kg, 4 doses), while no signs of in vivo toxicity were visible in rat with **12** up to 250 mg/kg. The high concentrations at which mitochondrial toxicity is observed in these ex vivo systems are, however, unlikely to be relevant for a systemic application in physiological systems. The observation of hepatic mitochondrial abnormalities in treated rats was the likely reason for discontinuation of clinical development of **12** by Sandoz. These alterations were not observed with the inactive enantiomer **13**. Together with the observations with oxirane carboxylic acids, these data point to the fact that it is CPT inhibition itself, and not an off-target effect of the single bioactive compounds, that causes mitochondrial dysfunction and alteration in mitochondrial morphology in these models. What remains unclear is the clinical relevance of these microscopical findings and the effects on a diseased organ, where there is an ongoing imbalance in the regulation of mitochondrial function that might actually be corrected by CPT inhibition. Macroscopically, treatment with **6a** has been associated repeatedly with increase in ALT (up to 2-fold). There is no indication as to whether this is an effect of the molecule or of the mechanism of action, possibly secondary to mitochondrial toxicity. In any case, discontinuation of the ERGO study by the biopharmaceutical company MediGene was declared to be either a consequence of this finding[257] or of insufficient efficacy.[258] As development of **6a** as an antidiabetic agent was also discontinued, there is reason to believe that liver safety was a concern in this context as well, although this finding is the object of some controversy.[259]

CPT1 is involved in spermatogenesis. Effects of CPT1B inhibitors on male fertility are therefore possible. Dietary **6a**, however, showed no effect on CPT1 activity in rat sperm.[260] Effects on fetal development are also a possible concern because of the neonatal phenotype of severe CPT2 deficiency.

Long-chain acylaminocarnitines have been reported to be extremely toxic upon ip administration probably because of their detergent-like properties.[114]

While a number of arguments support the possibility of establishing a reasonable therapeutic index for a partial noncontinuous CPT inhibitor, the data available raise a number of concerns that need to be properly addressed before an effective therapeutic agent based on CPT modulation can be made available to the patient. In particular, any clinical development plan must comprise relevant biomarkers to assess the safety of such intervention. Special attention should be paid to hepatic histopathology and serum markers of hepatic injury as well as to indices of muscle damage in the case of CPT1B and CPT2 inhibition, where cardiac function should also be monitored. It is important to note that many of the potential safety issues might not be relevant if partial and time-controlled CPT inhibition with low systemic exposure is achieved. An appropriate dose regimen, i.e., by postabsorptive inhibition ("bed-time application") might also be a relevant factor in the safety profile of CPT inhibitors. Additional reassuring evidence comes from the development compound **19**, a nonselective CPT2/CPT1A inhibitor. As detailed above, this compound was dosed in 40 T2D patients

for 15 days twice a day up to 450 mg/kg without any significant reported adverse events.

In conclusion, in the opinion of the authors none of the safety aspects discussed above preclude a priori the (re)assessment of CPT modulators in clinical setting, provided such concerns are adequately addressed in a clinical study plan.

CONCLUSION AND OUTLOOK

The origin of the long medicinal chemistry journey aimed to identify modulators of fatty acid oxidation may be traced to the seminal paper published in 1963 by Philip Randle when, in an attempt to describe fuel flux between and fuel selection by tissues, he coined the expression "glucose−fatty acid cycle".[192] Several investigators have confirmed the original Randle thesis and made it clear that depression of muscle glucose utilization and increased liver gluconeogetic rate, typically observed in people affected by diabetes mellitus, are the result of an enhanced lipid mobilization with high rates of fatty acid oxidation. Since then, an intensive hunt for potential inhibitors of fatty acid oxidation started. The first CPT inhibitor **1** and the related molecular species, although hampered by polypharmacology and lack of isoform specificity, offered convincing proof-of-concept data on the validity of such an approach to successfully treat the hyperglycemic condition in both the experimental model of T2D and the human diabetic patient.

It is notable that a considerable amount of animal and human studies have been conducted with compounds that can hardly be considered druglike according to modern standards. Although aliphatic epoxides are far less reactive than benzylic epoxides, the formation of irreversible bonds to proteins is not considered desirable nowadays because of the potential for formation of immunogenic conjugates. The carnitine derivatives, on the other hand, are permanently charged, zwitterionic entities with a heavy aliphatic component. The presence of zwitterionic or positively charged head (depending on the microenvironmental pH) is conducive to very poor oral bioavailability, as can be demonstrated in the case of **19**, where extensive pharmacokinetic studies have been conducted both in-house and by others. The "detergent-like" nature, although it does not seem to affect the integrity of the cellular membranes at therapeutic levels, is probably at the root of the observed dose-limiting effects on the GI tract (nausea, diarrhea) in humans. The mass of qualitative and quantitative pharmacological information that has been generated with these compounds and the fact that they have been associated with solid and convincing animal and human data and have merited attention as potential drugs should be a caveat against overinterpretation of the concept of "druglikeness" that permeates modern drug research.

More than 30 years after the description of the first synthetic CPT inhibitor, many questions remain: Is CPT modulation a viable pharmacological target? Why, despite the wealth of literature reports, have big pharma companies decided not to dedicate consistent efforts to the development of CPT modulators? Is the target "too old" to hold promise? On the other hand, is it not really a "new target", in view of the limited medicinal chemistry efforts that have been invested? Is there a perception that only substrate or malonyl-CoA analogues will affect CPT enzymes or that specific inhibition is not feasible? And above all, can this type of intervention, albeit doubtlessly effective in a number of possible indications, grant a sufficiently ample safety margin? While we may not be able to answer these questions in a satisfactory manner at this time point, it is beyond doubt that the use of CPT modulators so far has allowed the unveiling of the genetic ground and mitochondrial topology of the various isoforms of CPT and the deepening of the knowledge and understanding of the pathophysiology of cardiovascular and metabolic diseases and cancer. Moreover, we believe that the potential of this target to deliver both pharmacological tools and potential therapeutic modalities is far from being fully exploited.

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Odile Chomienne joined the Department of Metabolic and Cardiovascular Discovery at F. Hoffmann-La Roche in 1998, after doctoral studies at the University of Paris XI in France and postdoctoral studies at the University of Calgary in Canada in the fields of cardiovascular pharmacology and biochemistry. After 8 years of experience as Research Project Leader, she joined Clinical Development in 2006 as Global Project Manager and subsequently as a Group Leader in Project Management. At present, Odile is Group Leader in Project and Portfolio Management, specializing in research and clinical development of metabolic and cardiovascular projects.

Marcel Gubler received his Ph.D. in Life Sciences at the Federal Institute of Technology in Zürich (Switzerland) in 1988. During a postdoctoral fellowship at the Massachusetts Institute of Technology (Cambridge, MA), he conducted research in the area of metabolic engineering to optimize carbon flux into amino acid biosynthetic pathways in *Corynebacterium* sp. Subsequently, he joined Preclinical Research at F. Hoffmann-La Roche Ltd. (Basel, Switzerland), where he worked on novel targets for antibacterial therapies for several years. In 2000, he joined the Department of Metabolic Diseases to focus on the regulation of substrate utilization, fuel partitioning, and energy expenditure in mammalian cells with a particular interest in enzymology and pharmacological modulation of fatty acid synthesis and oxidation.

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■ ABBREVIATIONS USED

ACC, acetyl-CoA carboxylase; AIBA, aza-isobutyric acid; ALT, alanine transaminase; AUC, area under the curve; BHB, β-hydroxybutyrate; BW, body weight; CACT, carnitine—acylcarnitine translocase; CAD, coronary artery disease; CAT, carnitine acetyl transferase; CoA, coenzyme A; COT, carnitine octanoyl transferase; DNP, 2,4-dinitrophenol; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid) (Ellmann's reagent); FA, fatty acid; FAO, fatty acid oxidation; FBG, fasting blood glucose; FFA, free fatty acid; GIR, glucose infusion rate; GPT, glutamate pyruvate transaminase; GTT, glucose tolerance test; HFD, high fat diet; HGP, hepatic glucose production; icv, intracerebroventricular; ip, intraperitoneal; iv, intravenous; KB, ketone bodies = β-hydroxybutyrate + acetoacetate; LAD, left anterior descending; LCAC, long-chain acylcarnitine; LCFA, long chain fatty acid; LVDP, left ventricular derived pressure; MCR, metabolic clearance rate; MODY, maturity onset diabetes of the young; NIDD, non-insulin dependent diabetes; OGTT, oral glucose tolerance test; PDHC, pyruvate dehydrogenase; po, per os; ROS, reactive oxygen species; sc, subcutaneous; SD, Sprague—Dawley; STZ, streptozotocin; T1D, type 1 diabetes; T2D, type 2 diabetes; TG, triglyceride; WAT, white adipose tissue; BAT, brown adipose tissue; WAT, white adipose tissue; CHF, congestive heart failure

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(177) Background information on the performed assays can be found in the following: Jackson et al. *Biochem. J.* **1999**, *341*, 483−489 and Jackson et al. *J. Biol. Chem.* **2000**, *275*, 19560−19566. Human CPT1B and human and rat CPT1A and CPT2 cDNAs were subcloned in pGAPZB or pGAPZA, respectively. These plasmids were used to transform *P. pastoris* strain X-33 via electroporation after the preparation of electrocompetent cells. High copy number clones were selected where necessary using 0.5 or 1 mg/mL zeocin. Cultures for activity measurements were induced for 16 h in YPD medium (1% yeast extract, 2% peptone, 2% glucose). Crude cell extracts were prepared by disrupting the cells with glass beads or French press, depending on fermenter sizes. After centrifugation, the cell-free extracts were resuspended in cell breaking buffer (50 mM Tris, pH7.4, 100 mM KCl, 1 mM EDTA) in the presence of a protease inhibitor cocktail, before aliquoting and freezing at −20°C. CPT activity was measured using a spectrophotometric assay using 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), also called Ellman's reagent. The CoA-SH released on the formation of acylcarnitine from carnitine (500 μM) and palmitoyl-CoA (80 μM) reduced DTNB (300 μM), forming 5-mercapto-(2-nitrobenzoic acid) which absorbed at 410 nm with a molar extinction coefficient of 13 600 M^{-1} cm^{-1}. The assay buffer contained 120 mM KCl, 25 mM Tris, pH 7.4, and 1 mM EDTA.
(178) KB production was measured with cultured primary rat hepatocytes. Hepatocytes were isolated from fed normal Wistar rats by the liver collagenase-perfusion method. The cells were seeded in 24-well collagen-coated cell culture plates (250 000 cells per well) in RPMI 1640 medium without glucose containing 10% fetal bovine serum and 0.4% gentamycin, and the plates were incubated for 16 h at 37 °C. Medium was removed, and the cells were washed once with phosphate-buffered saline (PBS). Then 500 μL of fresh PBS containing 0.13% FA-free bovine serum albumin, 100 μM oleate, serial dilutions of CPT inhibitors, and DMSO up to 0.1% were added to the cells in each well, and incubation was continued for 6 h at 37 °C. At the end of the incubation, cell culture supernatants were collected and centrifuged and 40 μL samples were used for KB determination with a commercially available kit (Autokit 3-HB from Wako).
(179) FAO in primary rat and human hepatocytes was determined by measuring $^{14}CO_2$ release from ^{14}C-labeled palmitate. Hepatocytes were seeded in 96-well cell culture plates (50 000 cells/well) in 100 μL of William's E medium supplemented with 4 mM glutamine, 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.4% gentamycin, and the plates were incubated for 16 h at 37 °C. Medium was removed and the cells were washed once with PBS. Then 40 μL of prewarmed RPMI 1640 medium without glucose was added to each well and the plates were incubated for 2 h at 37 °C. An amount of 10 μL of serial dilutions of CPT inhibitors in glucose-free RPMI 1640 medium was added to each well, followed by addition of 50 μL of substrate mixture consisting of glucose-free RPMI 1640, 50 μM ^{14}C-labeled palmitate (0.4 μCi/mL), 100 μM L-carnitine, 0.1% FA-free bovine serum albumin, and DMSO up to 1%. The cell culture plates were covered with a 96-hole silicon gasket and with an inverted 96-well LumaPlate (PerkinElmer) whose scintillant coated wells were pretreated 1 day before use with 15 μL of strongly alkaline Soluene 350 solution (PerkinElmer). The culture plate/gasket/LumaPlate sandwich, held together by a custom-made clamp, was incubated at 37 °C for 4−6 h. $^{14}CO_2$ released during palmitate oxidation was captured by the alkaline scintillant in the LumaPlate, and after termination of the incubation, the sandwich was disassembled and the radioactivity in the LumaPlates was counted on a TopCount β counter (PerkinElmer).
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