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DOI: 10.1002/cmdc.200900535

Aminocarnitine Ureidic Derivatives as Inhibitors of Carnitine Palmitoyltransferase I

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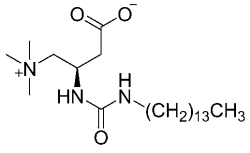
Type II diabetes is a complex metabolic disorder characterized by insulin resistance and impaired β-cell function.[1] It arises as a consequence of obesity, sedentary lifestyle and aging, with resulting hyperglycemia, blood pressure elevation and dyslipidemia. Moreover, in type II diabetes, highly increased hepatic fatty acid oxidation generates high levels of acetyl-coenzyme A (acetyl-CoA), ATP and NADH, which in turn upregulate gluconeogenesis and thus hepatic glucose production.[2] The transport of fatty acids into mitochondria is regulated by membrane-bound carnitine palmitoyltransferases (CPT) I and II.[3] CPT I, the outer mitochondrial membrane enzyme that is present as two isoforms known as liver (L-CPT I) and muscle (M-CPT I), catalyzes the formation of long-chain acylcarnitines. CPT II, the inner mitochondrial membrane enzyme present as a single isoform, converts long-chain acylcarnitines back into long-chain acetyl-CoA thioesters. CPT inhibitors, by lowering the level of acetyl-CoA, indirectly reduce liver gluconeogenesis.

Oxirane carboxylates, such as etomoxir and methyl 2-tetradecylglycidate,[4] previously identified as irreversible inhibitors of CPT, were found to induce cardiac hypertrophy due to a lack of liver and muscle isoform selectivity.

In previously published studies, Novartis (formerly Sandoz) described an alkylphosphate derivative of carnitine as a CPT I inhibitor,[5] and we described the identification of highly selective L-CPT I inhibitors.[6] Teglicar (**1**, ST1326) was chosen from these inhibitors for preclinical and clinical development as an antiketotic and antidiabetic agent.[6b]



Teglicar (**1**, ST1326)

The ureidic functional group present in teglicar was advantageous in terms of efficacy and selectivity towards the liver isoform of CPT, in comparison with other investigated moieties.[6] Taking these findings into consideration, we decided to continue our studies on L-CPT I inhibitors with a series of new aminocarnitine ureidic derivatives, exploring the effects of aromatic functionalities in the straight-chain alkyl group of **1**. The aim was to obtain new inhibitors with improved efficacy, while maintaining the high selectivity for the liver over the muscle isoform of CPT I, and/or with limited tensioactivity (an unwanted characteristic of this class of molecules arising from the long alkyl chain and ionic head). Oxygenated substitutions were introduced for their water coordinating properties, which could favorably limit packing of the molecules in micelle formation.

Accordingly, the compounds were synthesized starting from aminocarnitine and isocyanates or the corresponding carboxylic acids. For the most interesting molecule identified, the phosphonium analogue was also prepared in order to investigate the effects of ammonium group substitution with the bioisoster phosphonium on the activity profile.[7]

In order to explore the effects of aryloxy substituents in the alkyl chain of **1**, *ortho* and *meta* oxygen functionalities on an aromatic ring were inserted in derivatives **2**, **3** and **5**, in an attempt to obtain a lower packing of the molecules. These hexyloxy-phenoxyalkyl derivatives were prepared from carboxylic acids synthesized according to the standard procedures described in Scheme 1, subsequently transformed into isocyanates using diphenyl phosphoryl azide, or alternatively following classical activation as acyl chlorides, substitution with sodium azide and Curtius transposition. The phosphonium analogue **4** was also prepared following the same procedure, using (*R*)-4-trimethylphosphonio-3-aminobutyrate, prepared from D-aspartic acid as described in the literature,[8] instead of aminocarnitine.

Moreover, the effect of the aryl group adjacent to the ureidic functionality was explored in derivatives **6**, **10** and **11**, having an alkyloxy or a small alkyl chain as the substituent, and with a methylene group spacer between the ureido and aryl group, as in derivatives **7** and **8**, having an alkyloxy and/or a benzyloxy substituent. Conversely, a derivative with an aryloxy group at the end of a long chain was also prepared (compound **9**). All of these compounds were prepared starting from the isocyanate or the corresponding carboxylic acid, according to the procedure summarized in Scheme 2 (see Supporting Information for more details).

A three-dimensional homology model for human liver CPT I (hL-CPT I) was built using the crystallographic structure of murine carnitine acetyltransferase (CAT) co-crystallized with CoA and hexanoylcarnitine (PDB code: 2H3W). The sequence identity between the two enzymes is 33%. Moreover, the acyl pocket in human liver CPT I is characterized by an insertion of 14 amino acids (between 690 and 707), compared with other

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.200900535.

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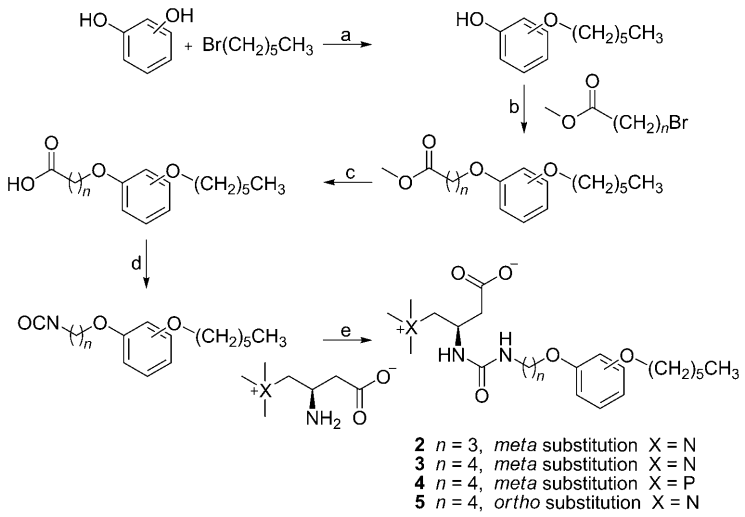
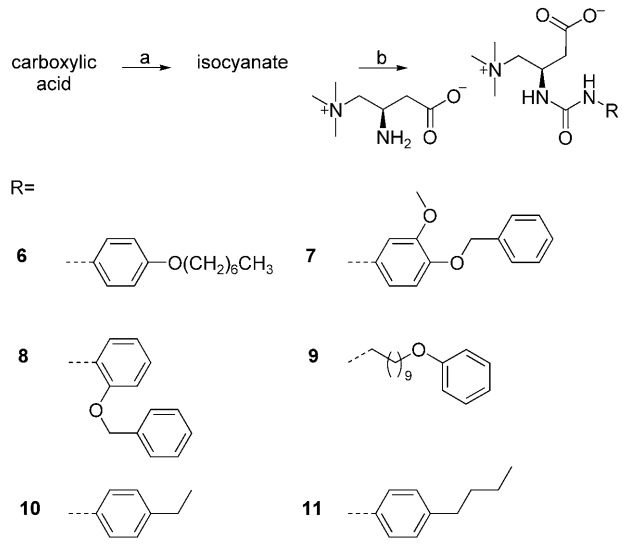


Scheme 1. Synthesis of compounds **2**–**5**. *Reagents and conditions*: a) NaH, anhyd DMF, 72 h, 80 °C, 97 %; b) NaH or Na_2CO_3, anhyd DMF, 18 h, 60 °C, 60–70 %; c) NaOH (2 N), MeOH/H_2O (2:1), 3 h, 50 °C→RT, 18 h, 55–92 % d) diphenylphosphorylazide, Et_3N, THF, reflux, 6 h; or $(CO)_2Cl_2$, CH_2Cl_2, 10 °C, 2 h; then NaN_3, toluene/acetone (3:1), 70 °C, 18 h; e) MeOH, RT, 24–48 h. Overall yield (steps d–e): **2**, 43 %; **3**, 20 %; **4**, 23 %; **5**, 47 %.



Scheme 2. Synthesis of compounds **6**–**11**. *Reagents and conditions*: a) diphenylphosphorilazide, Et_3N, THF, reflux, 6 h; or $(CO)_2Cl_2$, CH_2Cl_2, 10 °C, 2 h; then NaN_3, toluene/acetone (3:1), 70 °C, 18 h; or commercial corresponding isocyanate; b) MeOH, RT, 48 h. Overall yields (two steps): **6**, 55 %; **7**, 61 %; **8**, 57 %; **9**, 30 %; **10**, 90 %; **11**, 77 %.

model (98 % of residues in favored regions). A validation was performed by docking hexanoylcarnitine and compound **1** to the hL-CPT I model to verify the correct orientation of amino acids involved in relevant interactions in the active site.

In a score-based active/inactive separation study, the synthesized compounds were docked with the optimized hL-CPT I model. The alkyl chains were truncated to a maximum of ten atoms to reduce conformational bias. The resultant molecules were ranked into two classes by a Glide scoring function; in agreement with the observed biological activities (see below), compound **3** obtained the highest score (see Figure 1; data shown in the Supporting Information). These results are also useful for the further validation of the three-dimensional model, which could be used to design further generations of inhibitors.

All the prepared compounds were evaluated in vitro for their inhibition of L-CPT I, while only those compounds that exhibited improved efficacy over teglicar (**1**) against L-CPT I were evaluated for M-CPT I (heart) inhibition (a lower activity on M-CPT I compared with L-CPT I is desirable to avoid unwanted cardiac effects).[9,10] The IC_{50} values, determined under the same experimental conditions, for teglicar (**1**) and compounds **2**–**11** are reported in Table 1.

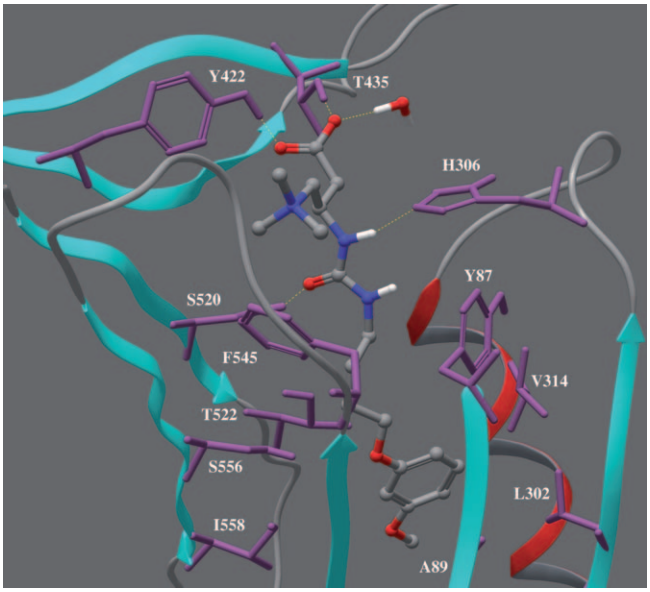


Figure 1. Orientation of compound **3** (atoms color coded by type) in the hL-CPT I model (relevant amino acids are in purple) constructed from the co-crystal structure of murine carnitine acetyltransferase (CAT) with CoA and hexanoylcarnitine (PDB code: 2H3W).

carnitine enzymes, such as CAT, carnitine octanoyltransferase (COT) and CPT II. Unfortunately the co-crystallographic structure of rat CPT II in complex with **1** (PDB code: 2FW3) could not be used to improve our hL-CPT I model because the acyl pocket of CPT II orients hydrophobic chains in a different environment due to the steric hindrance caused by the Met 135 residue (Ala 256 in hL-CPT I). A refinement procedure of the catalytic pocket was done within 15 Å from hexanoylcarnitine, and a ramachandran plot confirmed the good reliability of the

The insertion of the aryloxy group in the middle of the chain with two oxygen atoms in the *meta* position resulted in the highly active aminocarnitine derivatives **2** and **3**. In this case, the aromatic moiety binds in a large hydrophobic pocket formed by Val 314, Ile 558 and Leu 302. In particular, the resorci-

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Table 1. In vitro evaluation of CPT I inhibition.

Compd	IC_{50} [μM]	
	L-CPT I	M-CPT I
1 (teglicar)	0.68 ± 0.13	50.1 ± 5.90
2	1.40 ± 0.11	–
3	0.13 ± 0.03	5.44 ± 0.35
4	0.12 ± 0.05	57.3 ± 6.31
5	30.8 ± 2.31	–
6	> 100	–
7	> 100	–
8	> 100	–
9	0.49 ± 0.04	4.09 ± 0.33
10	> 100	–
11	> 100	–

Data are as the means of three determinations in duplicate ± SD. For compounds with an efficacy on liver isoform lower then the reference compound, teglicar (**1**), inhibition on muscle isoform was not evaluated (–).

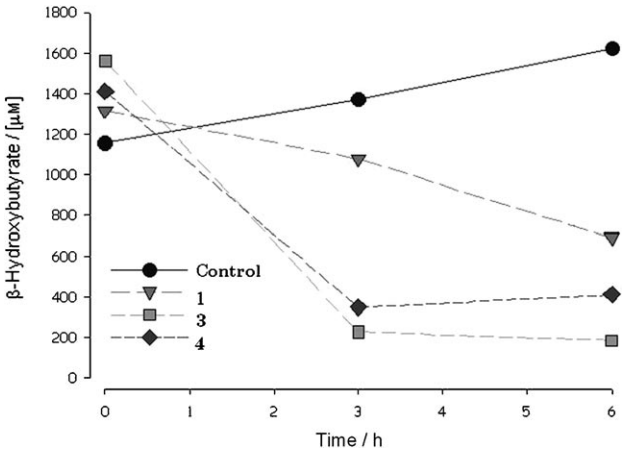

Figure 2. β-Hydroxybutyrate levels in fasted rats (17 h) treated with compounds **1** (reference), **3** and **4**. β-Hydroxybutyrate levels were measured at 3 and 6 h from single oral treatment (doses equimolar to 10 mg kg^{-1} of **1**; $n = 5$/group).

nol moiety of compound **3** binds such that one of the oxygen atoms is in proximity to Ser 556, leading to a good dipolar interaction that is not present in other derivatives. Compound **3** was found to be more active than teglicar (**1**), while maintaining very good liver/heart selectivity. Substitution of the ammonium group for a phosphonium group gave compound **4**, which exhibited a similar profile in vitro. *Ortho* substitution gave the least effective derivative (**5**). Molecular modeling studies using our hL-CPT I model indicated that the alkoxy group of the aryl moiety is unfavorably inserted into the catalytic pocket due to steric clashes with Ile 558. The aryl group directly connected to the urea (derivatives **6**, **10**, **11**) or spaced by a methylene (derivatives **7, 8**) did not result in active compounds. Docking studies suggest that the aromatic portion for these compounds binds in a polar pocket formed by Ser 556, Tyr 87 and Phe 545, and the number of bad contacts (ratio between distance and the sum of van der Waals radii up to 0.89) between the inhibitor and proteins is high. When the phenoxy group was present at the end of the chain (compound **9**), good inhibitory activity was observed, but with a partial loss in liver/heart selectivity.

In vivo results with selected compounds **3** and **4**, evaluated for their antiketotic and antidiabetic activity on fasted rats and diabetic mice, respectively, confirmed the improved efficacy of these compounds over teglicar (**1**). The antiketotic activity of compounds **3** and **4** was evaluated by measuring the inhibition of β-hydroxybutyrate production in fasted rats at doses equimolar to 10 mg kg^{-1} of **1**. The observed reduction of β-hydroxybutyrate production was higher and faster with both compounds **3** and **4** compared with **1**, reaching minimum values after 3 h that remained stable for an additional 3 h (Figure 2).

For compound **3**, inhibition of ketone bodies production was also evaluated at 0, 1, 3, 7, 10 mg kg^{-1} in rats fasted for 16 h, following the reduction of β-hydroxybutyrate for 9 h after a single oral administration. ED_{50} values, calculated on the basis of the area under the curve (AUC) from time 0 to 9 h, was equal to 3.7 mg kg^{-1}, lower than that found for **1** (ED_{50} = 14.5 mg kg^{-1}), and a faster onset of action was also observed (see figure S2 in the Supporting Information).

For antihyperglycemic activity evaluation, compounds **3** and **4** were orally administered to diabetic (db/db) mice once daily (30 mg kg^{-1}) for 13 days, using compound **1** as reference compound at a higher daily dose (80 mg kg^{-1}). Animals were observed daily throughout the experiment, and no changes in their general state of health or behavior were observed. At the end of the treatment, serum glucose levels were evaluated after 8 h fasting and 32 h from last administration. The results reported in Table 2 show that compound **3** induces a significant reduction (41%) in glucose levels. Compound **4** gave a slightly weaker effect (30%) at the same dose, and compound **1** induced only a 26% reduction in glucose levels, in spite of the much higher dosage.

Table 2. Antihyperglycemic activity in type II diabetic db/db mice.[a]

Compd	Dose [mg kg^{-1}]	Glucose [mg dL^{-1}]	P[b]
Vehicle control (10 mL kg^{-1} water)		708 ± 72	–
1	80	521 ± 119	< 0.01
3	30	418 ± 114	< 0.001
4	30	492 ± 108	< 0.05

[a] Mice were treated for 13 days. Data represent the mean ± SD ($n = 7$). [b] ANOVA test followed by Student-Newman-Keuls test; P values versus control.

It is worth remarking that in an insulin tolerance test (ITT) performed on day 12 on the same animals, under fed conditions (1 h fast) 6 h after the last treatment, a significant reduction in glucose AUC was observed in all treatment groups (~30%) with respect to the control, indicating an apparent increase in insulin sensitivity after prolonged L-CPT I inhibition.

As tensioactivity is an important parameter, and a potentially problematic physical property of compounds designed for oral administration, experimental critical micelle concentrations

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(CMC; higher CMC = lower tensioactivity) of the most promising compounds (those with $IC_{50} < 1\ \mu M$ against L-CPT I) were determined in comparison with compound **1**. Very similar values were found for **3** (7.9×10^{-4}), **4** (5.5×10^{-4}) and **9** (4.9×10^{-4} M), while compound **1** gave a value of approximately one order of magnitude lower (2.9×10^{-5} M). The lower CMC values observed for compounds **3** and **4**, probably due to the presence of the aryloxy moiety as in the case of **9**, contribute to their improved biological profiles compared with compound **1**.

In conclusion, these studies on CPT inhibitors have identified new active analogues of teglicar (**1**), the previously selected lead compound that is still under investigation. Among the new compounds, **3** and **4** appear to possess interesting properties in terms of in vitro (IC_{50} values) and in vivo profile; these compounds also possess desirable tensioactive properties (higher CMC, lower tensioactivity). Compound **3** was selected as a promising candidate for further investigations as an antidiabetic and/or antiketotic agent.

Experimental Section

The experimental protocols and characterization data for all intermediates and final products are reported in the Supporting Information together with molecular modeling, critical micelle concentration determination, and in vitro and in vivo biological experimental details.

The authorization to use animals in Sigma-Tau laboratories was obtained from the Italian Health Authority. Care and husbandry of animals is in accordance with European Directives no. 86/609 and with the Italian Regulatory system (D.L.vo no. 116, Art. 6; January 27, 1992). All parts of this study concerning animal care were approved by the official Sigma-Tau Veterinarian.

Acknowledgements

The authors wish to thank Dr. Maria Grazia Cima, Dr. Marco Quaglia, Gianfranco D'Amore, Roberto Catini, Emanuele Pani and Bernardino Ciani for their technical assistance, and Dr. Pierino Chiodi for his support in data elaboration and statistical evaluation.

Keywords: carnitine palmitoyltransferases · carnitines · inhibitors · teglicar · type II diabetes

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Received: December 24, 2009
Revised: February 22, 2010
Published online on March 15, 2010

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