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Article

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Development of Fluorinated Analogues of Perhexiline with Improved Pharmacokinetic Properties and Retained Efficacy

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ABSTRACT

We designed and synthesized perhexiline analogues that have the same therapeutic profile as the parent cardiovascular drug, but lacking its metabolic liability associated with CYP2D6 metabolism. Cycloalkyl perhexiline analogues **6a-j** were found to be unsuitable for further development as they retained a pharmacokinetic profile very similar to that shown by the parent compound. Multi-step synthesis of perhexiline analogues incorporating fluorine atoms onto the cyclohexyl ring(s) provided a range of different fluoro-perhexiline analogues. Of these, analogues **50** (4,4-*gem*-difluoro) and **62** (4,4,4',4'-tetrafluoro) were highly stable and showed greatly-reduced susceptibility to CYP2D6-mediated metabolism. In vitro efficacy studies demonstrated that a number of derivatives retained acceptable potency against CPT-1. Having the best balance of properties, **50** was selected for further evaluation. Like perhexiline, it was shown to be selectively concentrated in the myocardium and, using the Langendorff model, to be effective in improving both cardiac contractility and relaxation when challenged with high fat buffer.

INTRODUCTION

The therapeutic potential of perhexiline **1** (PHX, Fig. 1) in the treatment of cardiovascular disorders has been known for almost 50 years[1,2] and the drug was introduced in 1973 for the treatment of angina,[3] for which it has considerable efficacy and indeed continues to be used in some centres for patients with refractory angina. Perhexiline shifts myocardial metabolism from fatty acid to carbohydrate utilisation, thereby increasing metabolic efficiency (i.e. it produces relatively more ATP for a given oxygen consumption). By increasing myocardial efficiency, perhexiline has the potential to treat a range of disorders in which impaired cardiac energetics or oxygen deficiency play a role. In addition to angina, perhexiline has been investigated and used off-label for the treatment of heart failure, giving unprecedented improvements in $VO_{2\ max}$, quality of life improvement and left-ventricular ejection fraction;[4] for acute coronary syndromes;[5] inoperable aortic stenosis[6] and hypertrophic cardiomyopathy.[7] The latter study has led the FDA to award orphan drug status to perhexiline. Furthermore, by its inhibitory action on fatty acid β-oxidation, it has been hypothesised that perhexiline may have potential in anti-neoplastic therapies to target tumour metabolism, either alone or in combination with existing therapeutic regimens (*e.g.,* to increase susceptibility to chemotherapy).[8,9]

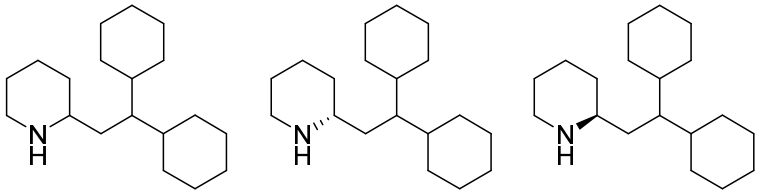


Figure 1. Perhexiline **1** and its enantiomers.[10]

However, perhexiline was removed from most markets by 1988, due to side-effects including hepatotoxicity and neurotoxicity.[11] The side-effects leading to withdrawal of the drug were

due to sustained high plasma levels of the drug, in a small subgroup of patients, as a result of variable (apparently unpredictable) metabolism. This led to phospholipid accumulation in the liver and peripheral nerves, due to complete blockade of mitochondrial long chain fatty acid uptake. Metabolism of perhexiline, hydroxylation at the 4-positions of either of the cyclohexyl rings, is mediated primarily by CYP2D6. CYP2D6 is polymorphic, giving substantial inter-individual variation in the rate of metabolism of drugs upon which it acts. An approximate 100-fold range is seen in the rate of perhexiline clearance and the required dosage ranges from 100 mg/week to 500 mg/day.[12] These issues can be avoided with close patient monitoring,[11] but such monitoring is not practical outside well-controlled patient groups, and thus it is unlikely that perhexiline can ever again be used in a wider patient population. Recent studies investigating the potential of using pure enantiomers **1a,b** (Fig. 1) of perhexiline have suggested differences in myocardial uptake, clearance, route of metabolism and, potentially, activity and toxicity; however, it is not yet clear as to whether these data will translate to therapeutic relevance.[13,14,15]

Perhexiline has been described as a drug of “low potency, low specificity, and low selectivity” and as having actions on a diverse range of molecular targets, some of which remain to be properly defined, which contribute to its therapeutic benefit in heart failure (HF).[16] However, at present, the therapeutic action of perhexiline is generally still thought to be due to inhibition of CPT-1 and to a lesser extent CPT-2.[11,17] CPT is an enzyme responsible for mediating mitochondrial uptake of long chain fatty acids, by coupling them to carnitine; the acylated carnitine is then taken into the cell by a carnitine acyl-carnitine translocase. Preventing free fatty acid transport across mitochondrial cell membranes causes a corresponding shift to greater carbohydrate usage and an increase in myocardial efficiency (10 - 40%; more in some studies,[11] in terms of ATP generated per unit of oxygen). This oxygen-sparing effect is of particular significance in the treatment of angina and heart failure, allowing cardiac output to remain constant in spite of reduced cardiac oxygen extraction. Perhexiline inhibits CPT-1 with modest potency (IC_{50} = 77 μM and 148 μM in rat cardiac and

liver mitochondria) and inhibits CPT-2 (79 μM) and fatty acid oxidation (β-oxidation) in hepatocytes at 22 μM, resulting in increased lactate and glucose utilisation.[18] Whilst the effective contribution of CPT inhibition has also been questioned,[15,16,18] a further argument in support of the importance of CPT-1 inhibition as the mechanism of action, in spite of the apparent lack of potency, is the tendency for perhexiline to become concentrated in tissues, including the myocardium.[18] Thus the concentration in the vicinity of mitochondrial CPT-1 is thought to exceed the modest IC_{50}. This characteristic is attributed to its amphipathic nature,[11] though this has also been highlighted as the source of the proposed diverse actions of perhexiline[16] and may also complicate attempts to determine the role of stereoselectivity in the therapeutic activity of perhexiline.

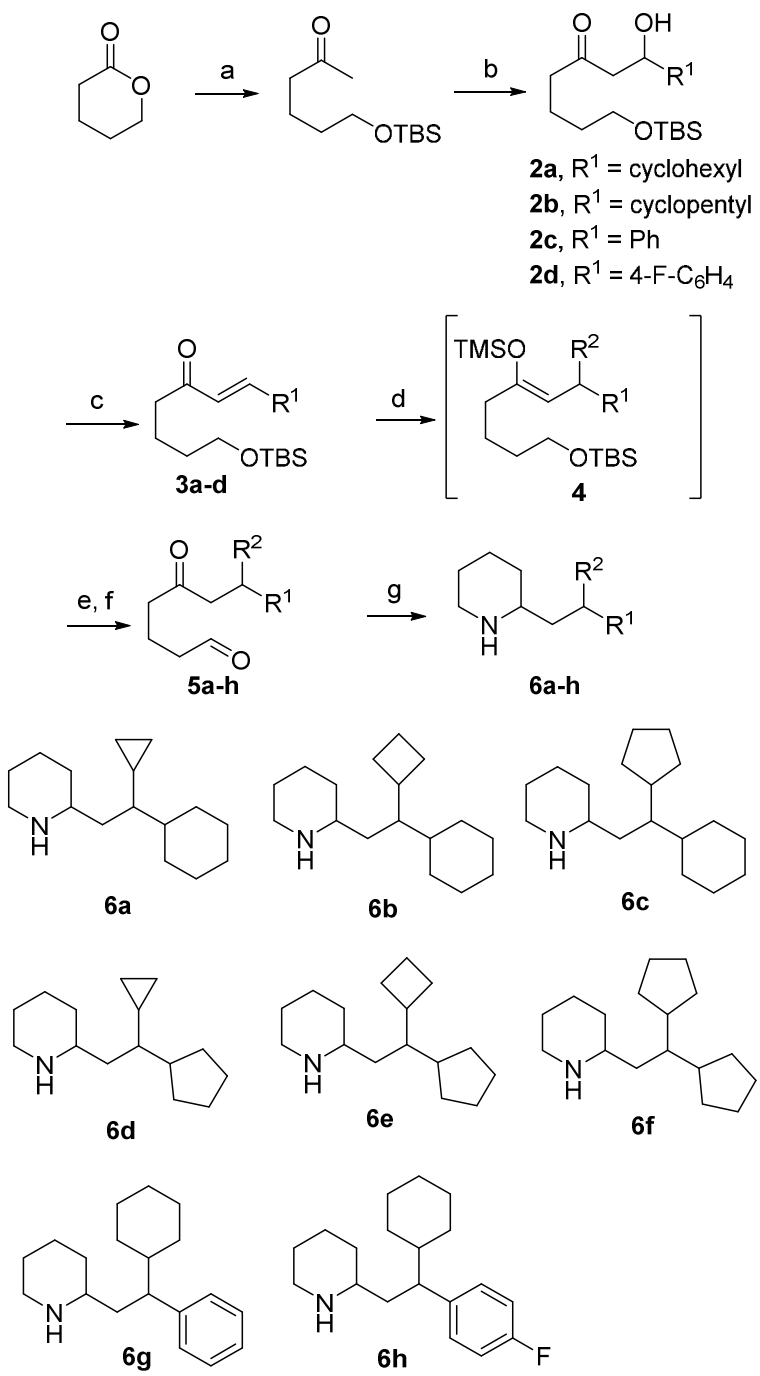
In spite of the therapeutic potential and the well-understood toxicity issues, which led to its removal from clinical usage throughout most of the world, few attempts have been made to modify the chemical structure of perhexiline. To date, only a small number of reports on derivatives have been published[19,20] and derivatives with improved properties (relative to perhexiline) have not been reported. It is clear that compounds which have the same therapeutic profile as perhexiline, but which lack the metabolic liability associated with CYP2D6 metabolism, could be highly effective therapeutic agents for the treatment of a range of cardiovascular conditions. In the absence of complex and variable pharmacokinetics, such a compound would not be restricted to limited patient populations or to use in a clinical environment, and thus could benefit a wider patient population for a range of cardiovascular diseases. In this report we describe the successful blocking of CYP2D6-mediated metabolism by addition of fluorine atoms at the sites of metabolism and demonstrate that these compounds have broadly equivalent potency to that of perhexiline against CPT-1.

RESULTS AND DISCUSSION

Chemistry and in vitro pharmacokinetics. In the absence of satisfactory hypotheses concerning how perhexiline exerts its therapeutic and toxicological actions, there is no clear guidance as to how the properties could be optimised. For example, there is no evidence that a more potent CPT-1 inhibitor would necessarily be preferred. Thus, our primary aim for this programme was to develop compounds having the same activity profile as perhexiline, but lacking the CYP2D6 liability. The obvious route to achieve this was the development of compounds which were structurally as similar to perhexiline as possible, in the hope that the therapeutic effects could be decoupled from the metabolic liability. There have been surprisingly few attempts made previously to generate even simple perhexiline analogues. These have included *N*-substituted derivatives,[20] derivatives in which the piperidine has been replaced by other amine-bearing groups,[19] derivatives in which the carbon separating the two cyclohexyl groups has been substituted with a carbinol group[21] and the synthesis of a deuterated derivative has also been described.[22]

As perhexiline can be hydroxylated on either cyclohexyl ring by CYP2D6, our initial focus was on the synthesis of analogues in which one or both of the cyclohexyl rings had been replaced by cycloalkyl rings of similar size (Scheme 1).

δ-Valerolactone was treated with methyl lithium and the resulting 6-hydroxy-hexan-2-one was transformed into the corresponding TBS-ether. The latter was treated with LDA and subjected to aldol reaction with aldehydes affording the β-hydroxy-ketones **2a-d**. The crotonic adducts **3a-d** were obtained by DBU-promoted β-hydroxy elimination of the intermediate mesylates. Introduction of the second group of the perhexiline-like structure was achieved by Michael reaction upon treatment of **3** with the corresponding cuprates,[23] then the intermediate silyl enol ethers **4** were deprotected and the primary carbinol group was oxidized with Dess-Martin periodinane (DMP) to provide the aldehydes **5a-h** (62 – 81% yields). The latter were subjected to reductive amination/cyclization[24] affording the small library of perhexiline analogues **6a-h**, which were isolated as mixtures of stereoisomers in good to excellent yields (78 – 91%).



Scheme 1.Synthesis of cycloalkyl perhexiline analogues **6a-h**. Reagents and conditions: (a) MeLi, -78 °C, then TBSCl and imidazole (93%); (b) LDA, -78 °C, then R^2CHO; (c) MsCl, TEA then DBU; (d) RM (M = Mg or Li), Cu(I)X (see experimental section), TMSCl, -78 °C; (e) PTSA, MeOH- CH_2Cl_2; (f) DMP (62-81% over steps d-f); (g) NH_4OAc, $NaBH_3CN$, MeOH (78-91%).

The THP analogue **6i** and the *gem*-dimethylcyclohexyl analogue **6j** (Fig. 2) were prepared following different synthetic routes (see Supporting Information for details).

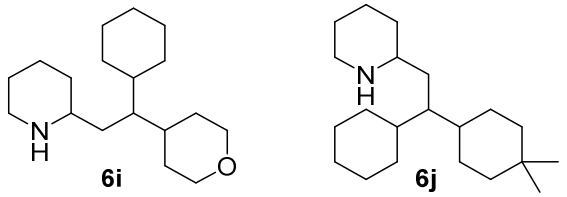
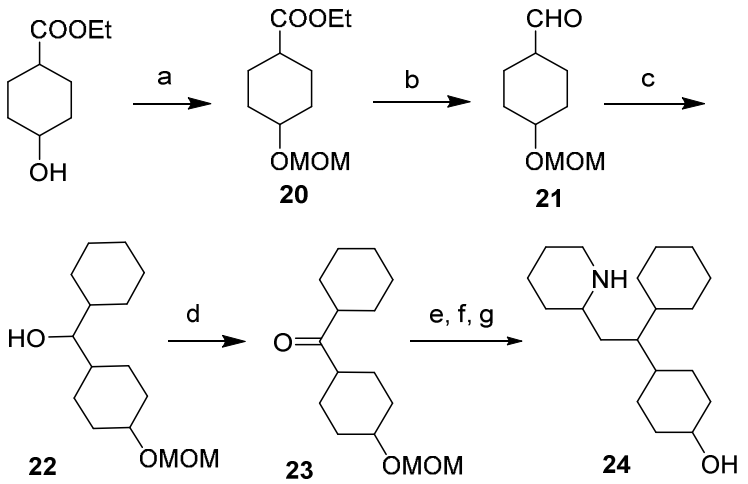


Figure 2.

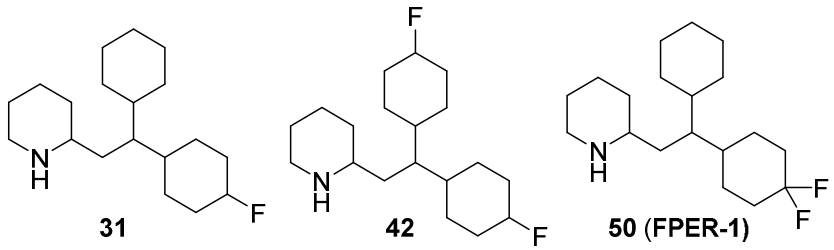
Disappointingly, in human and rat liver microsomal (HLM / RLM) stability studies, compounds **6a-j** all retained a stability profile very similar to that shown by the parent compound. We therefore turned our attention to the addition of fluorine atoms onto the cyclohexyl ring, as another potential route towards either physically blocking the site of metabolism, or to deactivating the ring towards oxidation by reduction of electron density.[25]

With the view of introducing fluorine by dehydroxyfluorination, we first synthesised 4-hydroxy-perhexiline **24** (Scheme 2) from ethyl 4-hydroxycyclohexane-carboxylate.



Scheme 2. Synthesis of 4-hydroxy-perhexiline **24**. Reagents and conditions: (a) MOMCl, DIPEA, CH_2Cl_2 (93%); (b) Aq. NaOH, then $NaBH_4$, THF, H_2O, then Swern oxidation (87% over three steps); (c) Cyclohexyl magnesium bromide, Et_2O (91%); (d) $Py_2Cr_2O_7$, CH_2Cl_2 (83%); (e) *n*BuLi, 2-picoline then $SOCl_2$; (f) PtO_2, H_2 (1 atm), MeOH; (g) TFA, CH_2Cl_2 then NaOH (82% over steps e-g).

The synthesis of fluorinated perhexiline derivatives proved unexpectedly difficult and the standard late-stage fluorination approaches were not successful in introducing a fluorine onto position 4 of the cyclohexyl ring. In fact, nucleophilic attack on a 4-cyclohexyl mesylate or triflate derived from dicyclohexyl-carbinol **14** (see Supporting Information, Scheme S2) using TBAF or KF led to elimination rather than substitution, while use of DAST to effect the dehydroxyfluorination of **24** (Scheme 2) – also in *N*-protected form - led to dehydration. An alternative approach, hydrogenation of the potential 4-fluorophenyl precursor **6h** (Scheme 1) using a number of different catalysts led predominantly to concomitant reductive defluorination and gave perhexiline as the end product. Attempts to access vinyl-fluoride **27a** (See Scheme S3, Supporting Information) via Ag-mediated *ipso*-fluorodestannylation of the corresponding vinyl stannane with electrophilic fluorinating agents resulted in impractical outcomes.[26] Eventually, we were able to develop multi-step synthetic strategies capable of producing enough pure fluoro-perhexilines **31**, **42**, **50**, **55** and **62** (Fig. 3) for performing biological tests.



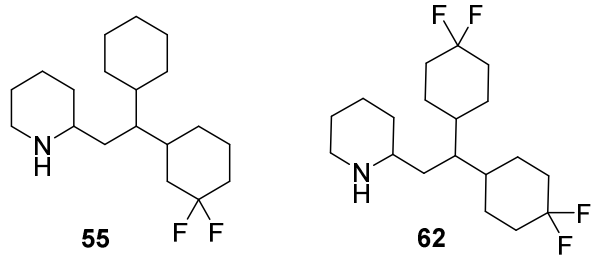
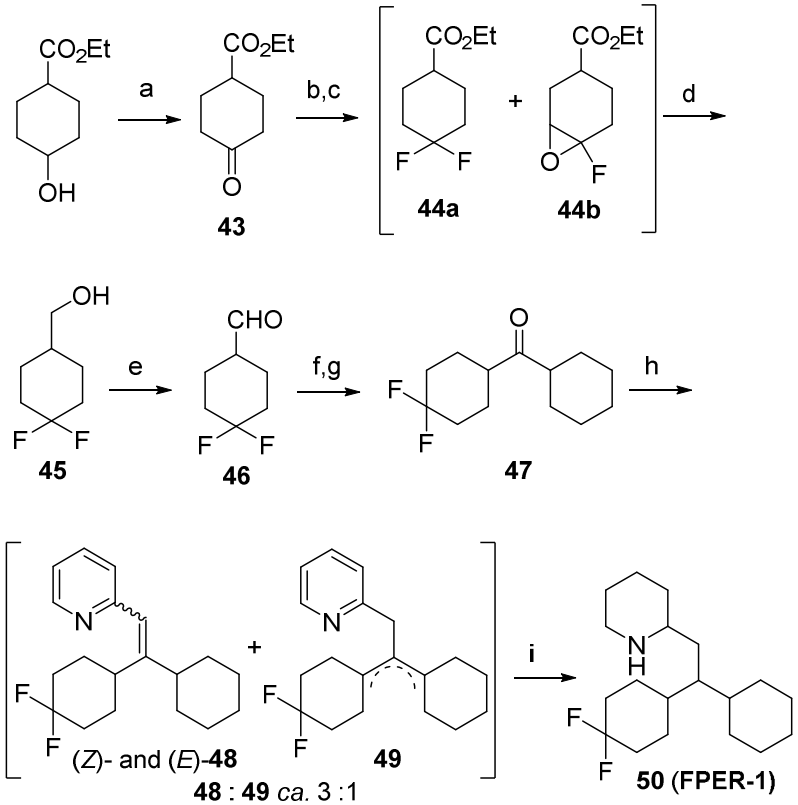


Figure 3. Fluoro perhexiline derivatives synthesised in this work.

As an example, the synthesis of 4,4-difluoro-perhexiline **50** (FPER-1) is shown in Scheme 3 (for detailed information on the synthesis of all fluoro-perhexilines see the Supporting Information). Commercially available ethyl 4-hydroxycyclohexane-carboxylate was first oxidized to ketone **43** and then submitted to deoxofluorination with DAST, affording an inseparable mixture of the desired *gem*-difluoro-ester **44a** and vinyl-fluoride by-product, which – for ease of separation - was oxidized with mCPBA to give the fluoro-oxirane **44b**. This mixture was then reduced with $LiAlH_4$, then FC purification afforded the difluoro-alcohol **45**, which was transformed into the target *gem*-difluorinated molecule **50** in 5 steps.

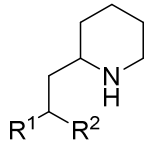


Scheme 3. Synthesis of 4,4-difluoro-perhexiline **50** (FPER-1). Reagents and conditions: (a) PCC (88%); (b) DAST; (c) mCPBA; (d) $LiAlH_4$ (62% over steps b-d); (e) Swern oxidation (64%); (f) Cyclohexyl magnesium chloride; (g) DMP (86% over steps f-g); (h) *n*BuLi, 2-picoline then $SOCl_2$ (88%); (i) $Pd(OH)_2/C$, H_2 (1 atm), AcOH, 50 °C (94%).

Racemic perhexiline **1** shows reasonable stability in the HLM assay, with a half-life of 100 minutes, but with a short half-life in RLM of just 21 minutes (Table 1). The relevance of this was validated in the presence of CYP2D6-expressing Bactosomes™, in which perhexiline was very rapidly turned over, with a half-life of just 14 minutes. As these rat liver microsomes are prepared from Sprague-Dawley rats, regarded as a model for CYP2D6 extensive metabolisers,[12] this is a logical correlation. The closest analogue of perhexiline **6c**, in which one cyclohexyl had been replaced by a cyclopentyl ring, showed near identical stability to the parent compound in both the RLM and CYP2D6 assays. As shown in Table 1, none of these unsubstituted cycloalkyl analogues **6a-j** showed significantly improved RLM stability compared to perhexiline **1**. The improved stability of "(+)-perhexiline" (absolute stereochemistry not defined at that stage) has previously been proposed.[27] The results shown in Table 1 may be taken to suggest that (*R*)-(+)-perhexiline **1a** was more stable than (*S*)-(-)-perhexiline **1b** in the HLM assay (98 and 65 min, respectively), whilst the two enantiomers had the same stability in the RLM assay (10 and 11 min, respectively). However, in view of the apparent lower stability of both of the two enantiomers in comparison with the racemic parent compound **1** in the RLM assay (21 min; a highly unlikely, if not impossible occurrence) we do not feel that this assay can be used to draw any conclusions concerning relatively small differences in potency. However, we were able to conclude that replacement of one or both cyclohexyl groups with other cycloalkyl groups did not remove the CYP2D6 liability. Other modifications, including the addition of a *gem*-dimethyl onto one of the cyclohexyl rings (**6j**), or replacement of one of the rings with a 4-tetrahydropyranyl (**6i**), with a phenyl (**6g**), or with a 4-fluorophenyl (**6h**), did not lead to significant improvements in the PK profile. The 4-tetrahydropyran derivative **6i** gave an apparent gain in RLM stability, but this was not reflected by any improvement in stability to CYP2D6. These results suggested that it would be necessary to either replace both cyclohexyl rings, or to more strongly deactivate a remaining cyclohexyl ring in order to remove the metabolic liability. Disappointingly, and unexpectedly, the addition of one

fluorine (compound **31**) gave no improvement in stability and, equally surprisingly, the addition of one fluorine on each cyclohexyl (compound **42**) gave a compound which was chemically unstable on storage in pure form. All other compounds showed no decomposition on incubation for 1 h in pH 7.4 PBS at 37 °C. It is not clear as to what caused this instability, but this compound was not further evaluated. At the other end of the scale, as predicted, the tetrafluoro derivative **62**, in which all of the potential sites for metabolism, as identified by previous studies[28] had been blocked, was extremely stable. Perhaps most interestingly, the addition of a 4,4-difluoro group onto just one of the cyclohexyl rings (compound **50**) also gave a very substantial increase in stability over the parent compound, and stability in the HLM, RLM and CYP2D6 assays had increased to >500, 72 and 104 min respectively (Table 1). Our hypothesis, aimed at leaving as much of the parent structure intact as possible, was that one, but perhaps not both, of the cyclohexyl rings formed an essential binding interaction with the target. Thus a derivative might be more likely to retain activity if only one ring had been modified. With this in mind, we also prepared the 3,3-difluorocyclohexyl derivative **55**, feeling that the reduction in electron density from a more distant group could be sufficient to reduce metabolism, whilst at the same time perhaps interfering less with a binding interaction. However, this compound did not show the same improvement in stability. These data show that certain fluorinated perhexiline analogues are much less heavily metabolised by CYP2D6 than perhexiline and thus should avoid the inter-individual variability which compromises perhexiline therapy. Therefore, these compounds are expected to show more reliable and predictable pharmacokinetics and hence may also avoid the toxicity issues found with perhexiline.

Table 1. Human and rat liver microsomal (HLM / RLM) stability studies.



	R^1	R^2	HLM $t_{1/2}$ (min)	RLM $t_{1/2}$ (min)	CYP2D6 $t_{1/2}$ (min)

			± SEM	± SEM	± SEM
(±)-PHX **1**	Cyclohexyl	Cyclohexyl	100 ± 1	21 ± 1	14 ± 3
(+)-(*R*)-PHX **1a**	Cyclohexyl	Cyclohexyl	98 ± 25	10 ± 0.8	-
(–)-(*S*)-PHX **1b**	Cyclohexyl	Cyclohexyl	65 ± 9	11 ± 0.4	-
6a	Cyclohexyl	Cyclopropyl	124 ± 18	3 ± 0.5	-
6b	Cyclohexyl	Cyclobutyl	>200	10 ± 0.6	-
6c	Cyclohexyl	Cyclopentyl	>150	19 ± 2	16 ± 1
6d	Cyclopentyl	Cyclopropyl	27 ± 2	3 ± 0.04	-
6e	Cyclopentyl	Cyclobutyl	51 ± 5	7 ± 0.5	-
6f	Cyclopentyl	Cyclopentyl	50 ± 4	14 ± 1	-
6g	Cyclohexyl	Phenyl	56 ± 7	4 ± 0.3	-
6h	Cyclohexyl	4-Fluorophenyl	79 ± 8	7 ± 0.2	-
6i	Cyclohexyl	4-Tetrahydro pyranyl	84 ± 21	31 ± 1	7 ± 0.8
6j	Cyclohexyl	4,4-Dimethyl cyclohexyl	77 ± 11	20 ± 1	-
31	Cyclohexyl	4-Fluoro cyclohexyl	65 ± 7	11 ± 0.9	16 ± 0.5
50	Cyclohexyl	4,4-Difluoro cyclohexyl	>500	72 ± 6	104 ± 17
55	Cyclohexyl	3,3-Difluoro cyclohexyl	>150	35 ± 1	-
42	4-Fluoro cyclohexyl	4-Fluoro cyclohexyl	-	-	-
62	4,4-Difluoro cyclohexyl	4,4-Difluoro cyclohexyl	>4000	>1500	>150

Biology: We compared our compounds to perhexiline **1** in a suitable efficacy model in which perhexiline has shown potency. Compound selection was guided by first determining *in vitro* inhibitory potency against the most widely accepted target for perhexiline, CPT-1 (Table 2). Inhibitory potency against rat mitochondrial CPT-1 *in vitro* was measured by determining the ability of derivatives to inhibit the conversion of palmitoyl CoA and L-carnitine into palmitoylcarnitine, measuring the disappearance of palmitoyl CoA spectrophotometrically, as described previously.[29] In this assay the ~50% of the CPT activity that is sensitive to inhibition by malonyl CoA is defined as CPT-1,[29] and under the conditions of the assay we obtained an IC_{50} of 1.2 μM for malonyl CoA. Since all tested inhibitors also showed the same maximal inhibition of activity as malonyl CoA, we assumed that this also represents inhibition of CPT-1.

We found that the *gem*-difluoro derivative **50** showed only a small loss in inhibitory potency (45 μM, $P<0.05$) compared to racemic perhexiline **1** (11 μM) and its (*S*) enantiomer **1b** (14 μM). The apparent increased potency of the (*S*) enantiomer **1b** matches the published

suggestion that "(–)-perhexiline" might have better therapeutic properties.[14] The (*R*) enantiomer **1a** shows a significant reduction in potency compared to racemic perhexiline **1** (32 μM, $P<0.05$) and approaches significance when compared to the (*S*) enantiomer **1b**. This compares to a literature value of 77 μM for perhexiline **1**.[17] However, the difficulties in obtaining precise and consistent data for CPT-1 inhibition have been noted and discussed by Ceccarelli.[18] The exceptionally stable tetrafluoro derivative **62** had lost some of its activity against CPT-1 (88 μM, $P<0.05$ compared to **50**). Replacement of one cyclohexyl with a cyclopentyl in compound **6c** had no impact on potency (11 μM), whilst replacement of both cyclohexyls with cyclopentyl groups, in compound **6f**, gave a non-significant reduction in potency (19 μM). However, the hypothesis that retaining one intact cyclohexyl might be sufficient to retain activity, whilst a wide range of modifications on the other might be tolerated, was disproved by the loss of potency seen with the 3,3-difluoro **55** (215 μM) and tetrahydropyran **6i** (715 μM) derivatives. We also investigated the stereoisomeric mixture of 4-hydroxy derivatives **24** to study the possibility that the activity of perhexiline was in-part mediated through a metabolite,[28] but instead found it to be very poorly active (475 μM). Having the best balance of pharmacokinetic and inhibitory properties, plus relative ease of synthesis, we selected the 4,4-*gem*-difluoro **50** derivative to take forward for *in vivo* PK and efficacy study.

Table 2. CPT-1 inhibitory activities.

	R^1	R^2	IC_{50} for CPT-1 inhibition (μM)*
(±)-PHX **1**	Cyclohexyl	Cyclohexyl	11 (7 - 17)
(+)-(*R*)-PHX **1a**	Cyclohexyl	Cyclohexyl	32 (24 - 41)
(–)-(*S*)-PHX **1b**	Cyclohexyl	Cyclohexyl	14 (8 - 25)
6c	Cyclohexyl	Cyclopentyl	11 (5 - 24)
6f	Cyclopentyl	Cyclopentyl	19 (7 - 40)
6i	Cyclohexyl	4-Tetrahydropyranyl	715 (510 - 1050)
50	Cyclohexyl	4,4-Difluorocyclohexyl	45 (34 - 60)
55	Cyclohexyl	3,3-Difluorocyclohexyl	215 (160 - 290)
62	4,4-Difluorocyclohexyl	4,4-Difluorocyclohexyl	88 (61 - 127)
24	Cyclohexyl	4-Hydroxycyclohexyl	475 (290 - 745)

* mean and pooled 95% confidence interval of two independent experiments.

***In vivo* pharmacokinetics:** It has been proposed that the unexplained activity of perhexiline **1**, in spite of modest *in vitro* potency against all proposed targets, is due to selective accumulation in the myocardium. Thus, in a continued attempt to ensure that our compounds mirrored perhexiline in as many aspects as possible, we conducted studies to determine the *in vivo* stability and myocardial exposure. The distribution and stability of **50**, following daily oral dosing, were compared to perhexiline in male BALB/c mice (samples taken at 1, 4, 8 and 24 h following dosing; n = 3 per timepoint – see supplementary information for full details). Plasma and myocardial concentrations, and area under the curve, following 10 mg/kg oral gavage of **50** and perhexiline were investigated. In the mouse model, perhexiline **1** is very rapidly metabolised and thus no direct comparisons can be drawn between the absolute concentrations of the two compounds. However, Table 3 shows that there is a significant accumulation of perhexiline in the myocardium (5.7-fold at 1 h, 4.9-fold at 4 h and 4.8-fold at 8 h, as defined by drug concentration; 2.4-fold as defined by AUC), whilst **50** shows an even larger selective accumulation (10.7-fold at 1 h, 10.9-fold at 4 h and 9.5-fold at 8 h, as defined by drug concentration; 8.6-fold as defined by AUC).

Table 3. *In vivo* pharmacokinetics in a mouse model

		50 (10 mg/kg)		Perhexiline (10 mg/kg)	
		Plasma	Myocardium	Plasma	Myocardium
Conc (ng/mL)	1 h	346.5 ± 257.4	3726.8 ± 2808.5	116.4 ± 39.2	664.5 ± 157.5$^{\#}$
	4 h	171.9 ± 44.1*	1877.3 ± 606.1*$^{\#}$	28.3 ± 8.8	125.8 ± 62.5
	8 h	191.8 ± 48.0*	1826.7 ± 498.7*$^{\#}$	6.0 ± 5.6	29.2 ± 25.2
AUC 0-24 (hr*ng/mL)		3763 ± 1501*	32360 ± 7030**$^{\#\#}$	876 ± 123	2114 ± 265$^{\#\#}$

*P<0.05 relative to perhexiline; **P<0.01 relative to perhexiline; $^{\#}P$<0.05 relative to plasma; $^{\#\#}P$<0.01 relative to plasma (Student's t-test).

Further studies were conducted, showing that selective myocardial accumulation was also seen in male Sprague-Dawley rats (used as a model for CYP2D6-mediated metabolism)[12] (Table 4). The distribution and stability of **50** following daily oral dosing were compared to

perhexiline **1** (plasma samples taken at 1, 2, 4, 8 and 24 h following dosing; n = 3 per timepoint – see Supporting Information for full details). Again perhexiline is metabolised very quickly, which is not representative of the situation in humans, but similar myocardium : plasma ratios can be seen in comparing perhexiline **1** (19-fold at 1 h and 11.5-fold at 8 h, as defined by drug concentration) and **50** (15.3-fold at 1 h and 7.2-fold at 8 h, as defined by drug concentration).

Table 4. *In vivo* pharmacokinetics in a rat model

		50 (10 mg/kg)		Perhexiline (10 mg/kg)	
		Plasma	Myocardium	Plasma	Myocardium
Conc (ng/mL)	1 h	386.3 ± 28.4*	5913.4 ± 1350.2#	99.9 ± 43.5	1901.2 ± 887.1#
	2 h	375.4 ± 139.7*	-	112.9 ± 32.6	-
	4 h	297.1 ± 145.2	-	97.44 ± 24.3	-
	8 h	178.6 ± 93.4	1285.9 ± 822.8	31.4 ± 5.7	361.2 ± 104.7#

*P<0.05 relative to perhexiline; #P<0.05 relative to plasma (Student's t-test)

Thus, assuming that this accumulation is responsible for the therapeutic effect, rather than presenting a toxicological liability, **50** appears to match our requirements as an optimised (in terms of CYP2D6 liability) orally-bioavailable analogue of perhexiline, suitable for efficacy studies.

***Ex vivo* efficacy studies:** The *ex vivo* Langendorff murine model was used to assess the effects of perhexiline **1** and analogue **50** on indicators of cardiac contractility (LVdP/dt max) and relaxation (LVdP/dt min) following perfusion with a high fat buffer. In recent years it has been shown that HF can be characterised by severe metabolic disturbances, intrinsic to the myocardium, which include fatty acid overload and build-up within the cardiomyocyte cytosol and mitochondria.[30] As such, energy production within the cardiomyocyte is impaired, which in turn disrupts cardiac function and contractile activity over time. By perfusing hearts with a high fat buffer, we can simulate the fatty acid overload observed during HF and can measure

the effects of perhexiline **1** and compound **50** on cardiac function, such as LVdP/dt max and min, under these conditions.[31]

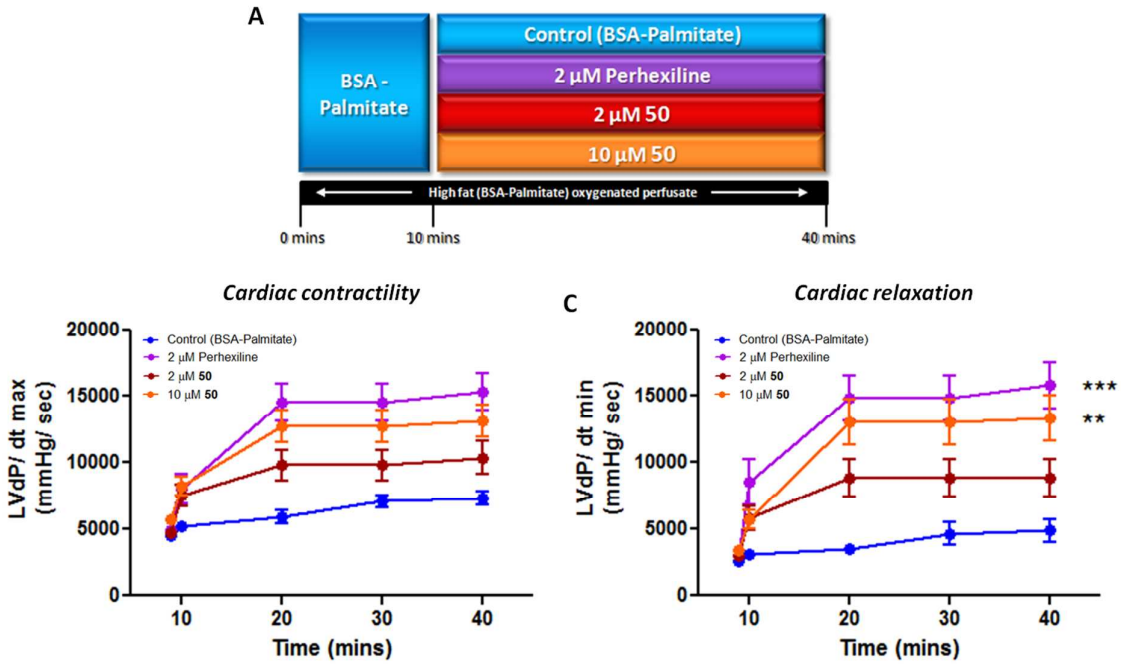


Figure 2. Perhexiline **1** and 4,4-*gem*-difluoro-perhexiline **50** improve cardiac contractility and relaxation in a high fat perfusate. A: The Langendorff perfusion protocol. Hearts were stabilised for 10 minutes with BSA-Palmitate and then perfused for 30 minutes with either 1) Control (BSA-Palmitate), 2) 2 µM perhexiline, 3) 2 µM **50** or 4) 10 µM **50**. Cardiac contractility (LVdP/dt max) and cardiac relaxation (LVdP/dt min) were calculated from the left ventricular pressure. **B:** LVdP/dt max. **C:** LVdP/dt min. Data are presented as mean ± SEM; n = 12-19 mice. ***P*<0.01 vs Control (BSA-Palmitate); ****P*<0.001 vs Control (BSA-palmitate) (1-way ANOVA, post-hoc Bonferroni test.

The results demonstrate that 2 µM perhexiline **1** shows a strong trend for enhancing LVdP/dt max (2.1 fold increase when compared to the control; n=16; *P*=0.07) and is highly effective for enhancing LVdP/dt min (3.2 fold increase when compared to the control; n=16; *P*<0.001) in the presence of high fat. At a concentration of 10 µM, like perhexiline, **50** showed a similar trend to increasing LVdP/dt max (1.8 fold increase when compared to the control; n=16

P=0.07) and was almost as effective as perhexiline in increasing LVdP/dt min (2.7 fold increase when compared to the control; n=16; *P*<0.01). These results also demonstrate that 2 μM perhexiline and 10 μM **50** have significant lusitropic effects, a feature that current pharmacological treatments for HF lack. It can be noted that the apparent 5-fold loss of potency of **50** compared to perhexiline is in accord with the relative potencies vs CPT-1; however - as it cannot be assumed that CPT-1 is the only target, or even the major target, or that the ratio of potencies for perhexiline and **50** vs other targets will remain the same – the importance of this observation cannot be determined. At present, most drug therapies for HF only improve systolic function such as cardiac contractility, and therefore only benefit patients with systolic HF.[32] As such, perhexiline and **50** show promising potential for use in diastolic HF, a condition in which cardiac relaxation is impaired, as opposed to contractility, and where there are currently no well-defined existing treatments.[32-34] Furthermore, **50** at 2 μM shows a small improvement on both cardiac parameters with clear evidence of dose dependency.

CONCLUSION

In summary, these results reveal that 4,4-*gem*-difluoro-perhexiline **50** (FPER-1) holds a similar therapeutic profile to perhexiline **1** and thus **50**, and possibly other analogues of perhexiline, may have the same potential for the treatment of HF and cardiovascular diseases, but without the metabolic liability of the parent compound. As a consequence, compounds with the properties exemplified by **50** are expected to be suitable for use across a broader spectrum of the patient population and not to require additional monitoring of drug plasma concentrations or patient phenotyping, both of which limit drug use and convenience. However, it is also clear from these studies that elucidation of the mechanism of action of perhexiline will be required before it will be possible to conduct an informed drug discovery programme to generate a perhexiline-like drug for clinical development.

EXPERIMENTAL SECTION

General Methods: All reactions were carried out under a nitrogen or argon atmosphere with dry solvents under anhydrous conditions, unless otherwise mentioned. Solvents and reagents: anhydrous tetrahydrofuran (THF), diethyl ether (Et_2O), methylene chloride (CH_2Cl_2) and toluene were purchased from commercial suppliers. Reagents were purchased at the highest commercial quality, used without further purification and handled in accordance with COSHH regulations. Chromatography: flash chromatography (FC) was carried out on silica gel (Merck Silica gel Si 60, 40-63 μm) according to the method described by Still.[35] Thin-layer chromatography (TLC) was carried out on glass-based 0.25 mm Merck silica gel plates (60F-254) which were developed with UV irradiation (254 nm and 365 nm), an aqueous solution of $KMnO_4$ and an ethanol solution of ammonium molybdate, and heat as developing agents. ^{1}H NMR spectra: these were recorded at 400 MHz on a Bruker ADVANCEIII 400 instrument. Chemical shifts (δ_H) are given in parts per million (ppm) as referenced to the appropriate residual solvent peak. ^{13}C NMR spectra: these were recorded at 101 MHz on a Bruker ADVANCE III 400 instrument. Chemical shifts (δ_C) are given in parts per million (ppm) as referenced to $CHCl_3$. ^{19}F NMR spectra: these were recorded at 376 MHz on a Bruker ADVANCE III 400 instrument without ^{1}H decoupling. Chemical shifts (δ_C) are given in parts per million (ppm) as referenced to $CFCl_3$ as 0 ppm. All NMR spectra were recorded at 298K unless otherwise stated. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad. Purities of all tested compounds were determined by reverse phase HPLC-MS using an Agilent 1200 series chromatograph system coupled with an Agilent G6120 signal quadrupole detector equipped with an electrospray ionisation source and detection in positive mode. HPLC conditions for purity analysis: Zorbax Eclipse Plus C18 column, 4.6 × 100 mm, 3.5 μm; mobile phase: 75% MeOH / 25% H_2O with 0.1% formic acid; flow rate: 1 mL/min. All tested compounds were found to be at least 96% purity. HRMS: analyses were performed using an LTQ Orbitrap XL MS spectrometer.

Representative procedures for carbocyclic perhexiline analogues.

6-((*tert*-Butyldimethylsilyl)oxy)hexan-2-one

A solution of MeLi (14.8 mL, 23.71 mmol, 1.6 M in Et_2O) was added at -78 °C to an Et_2O (10 mL) solution of δ-valerolactone (2.158 g, 21.55 mmol). The resulting reaction mixture was stirred for 1 h at -78 °C before quenching with NH_4Cl (10 mL, sat. aq.) and dilution with EtOAc (20 mL). The layers were separated and the organic layer was washed with brine (20 mL), dried (Na_2SO_4) and concentrated under reduced pressure to give the crude hydroxy ketone, which was used directly without further purification.

TBSCl (3.92 g, 26.0 mmol) and imidazole (2.31 g, 33.93 mmol) were added at 0 °C to a CH_2Cl_2 (10 mL) solution of the crude hydroxy ketone. The resulting mixture was allowed to warm to 23 °C and stirred for 3 h prior to quenching with a saturated solution of $NaHCO_3$ (10 mL) and dilution with CH_2Cl_2 (10 mL). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 5 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. FC (silica gel, hexanes:EtOAc 5:1) afforded 6-((*tert*-Butyldimethylsilyl)oxy)hexan-2-one (4.64 g, 93% for two steps) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.60 (t, *J* = 6.3 Hz, 2H), 2.44 (t, *J* = 7.3 Hz, 2H), 2.12 (s, 3H), 1.69 – 1.58 (m, 2H), 1.56 – 1.45 (m, 2H), 0.88 (s, 9H), 0.03 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 209.1, 62.8, 43.5, 32.2, 29.8, 25.9, 20.3, 18.3, -5.3. *m/z* (ESI) 231 $[M+H]^+$.

7-((*tert*-Butyldimethylsilyl)oxy)-1-cyclohexyl-1-hydroxyheptan-3-one (2a)

Method A: a solution of freshly prepared LDA (3.80 mL, 3.80 mmol, 1.0 M in THF/hexanes) was added at -78 °C to a THF (5 mL) solution of 6-((*tert*-butyldimethylsilyl)oxy)hexan-2-one (730 mg, 3.17 mmol). The resulting mixture was stirred for 1 h at -78 °C before cyclohexanecarboxaldehyde (426 mg, 460 µL, 3.80 mmol) was added. The reaction mixture was stirred at -78 °C for further 30 min before quenching with NH_4Cl (10 mL, sat. aq.) and dilution with EtOAc (10 mL). The layers were separated and the organic layer was washed

with brine (20 mL), dried (Na_2SO_4) and concentrated under reduced pressure. FC (silica gel, hexanes:EtOAc 2:1) furnished the aldol adduct **2a** (955 mg, 88 %) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.85 – 3.73 (m, 1H), 3.60 (t, *J* = 6.2 Hz, 2H), 3.04 (brs, 1H), 2.67 – 2.39 (m, 4H), 1.89 – 1.78 (m, 1H), 1.78 – 1.70 (m, 2H), 1.69 – 1.57 (m, 4H), 1.54 – 1.47 (m, 2H), 1.41 – 1.29 (m, 1H), 1.29 – 1.09 (m, 3H), 1.09 – 0.92 (m, 2H), 0.88 (s, 9H), 0.04 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 212.6, 71.7, 62.7, 46.1, 43.4, 43.0, 32.1, 28.8, 28.3, 26.4, 26.2, 26.1, 25.9, 20.1, 18.3, -5.3. *m/z* (ESI) 343 $[M+H]^+$.

(*E*)-7-((*tert*-Butyldimethylsilyl)oxy)-1-cyclohexylhept-1-en-3-one (3a)

Method B: Et_3N (540 µL, 3.87 mmol) and MsCl (150 µL, 1.94 mmol) were added at 0 °C to a CH_2Cl_2 solution of aldol adduct **2a** (440 mg, 1.28 mmol). The solution was stirred for 1 h at 0 °C followed by addition of DBU (290 µL, 1.94 mmol). The resulting mixture was warmed to 23 °C and stirred for further 2 h prior to quenching with NH_4Cl (10 mL, sat. aq.) and dilution with EtOAc (20 mL). The layers were separated and the organic layer was washed with brine (20 mL), dried (Na_2SO_4) and concentrated under reduced pressure. FC (silica gel, hexanes:EtOAc 5:1) afforded the enone **3a** (410 mg, 98%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 6.76 (dd, *J* = 16.0, 6.8 Hz, 1H), 6.05 (dd, *J* = 16.0, 1.4 Hz, 1H), 3.63 (t, *J* = 6.2 Hz, 2H), 2.57 (t, *J* = 7.3 Hz, 2H), 2.19 – 2.08 (m, 1H), 1.81 – 1.73 (m, 4H), 1.73 – 1.60 (m, 3H), 1.60 – 1.49 (m, 3H), 1.36 – 1.26 (m, 2H), 1.22 – 1.09 (m, 2H), 0.89 (m, 9H), 0.04 (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 201.1, 152.1, 127.8, 62.9, 40.6, 39.8, 32.3, 31.8, 25.95, 25.92, 25.7, 20.8, 18.3, -5.3. *m/z* (ESI) 325 $[M+H]^+$.

7-Cyclohexyl-7-cyclopropyl-5-oxoheptanal (5a)

Method C:[23] a solution of cyclopropylmagnesium bromide (1.67 mL, 1.67 mmol, 1.0 M in 2-MeTHF) was added at -78 °C to a suspension of CuI (159 mg, 0.835 mmol) in THF (2 mL). The resulting mixture was allowed to warm to 0 °C and stirred for further 1 h to give the

organocuprate reagent. The solution was then cooled to -78 °C followed by addition of a THF solution (0.5 mL) of enone **3a** (180 mg, 0.56 mmol) and TMSCl (142 µL, 1.12 mmol). The reaction mixture was stirred at -78 °C for 1 h before quenching with a MeOH solution of PTSA (2 *N*, 5 mL, 10 mmol). The solution was stirred at 23 °C for 2 h prior to dilution with EtOAc (20 mL). The layers were separated and the organic layer was washed with brine (10 mL), dried (Na_2SO_4) and concentrated under reduced pressure. The mixture was passed through a plug of silica gel (EtOAc) to give the corresponding alcohol, which was used without further purification.

Dess−Martin periodinane (285 mg, 0.67 mmol) was added at 23 °C to a CH_2Cl_2 solution (5 mL) of the alcohol above. The reaction mixture was stirred for 6 h before quenching with Na_2SO_3 (5 mL, sat. aq.). The resulting mixture was extracted with CH_2Cl_2 (3 × 5 mL), combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by FC (silica gel, hexanes:EtOAc 3:1) afforded the dicarbonyl compound **5a** (101 mg, 73 % for three steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.82 (t, *J* = 1.5 Hz, 1H), 2.59 – 2.39 (m, 5H), 1.78 – 1.42 (m, 8H), 1.42 – 1.26 (m, 1H), 1.26 – 0.91 (m, 6H), 0.68 – 0.49 (m, 1H), 0.49 – 0.37 (m, 1H), 0.37 – 0.22 (m, 1H), 0.12 (tt, *J* = 14.5, 7.2 Hz, 1H), -0.09 (td, *J* = 9.3, 5.3 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 212.1, 201.3, 46.0, 45.5, 43.4, 42.8, 32.1, 30.6, 30.1, 26.8, 26.74, 26.68, 19.8, 14.21 5.6, 3.4. *m/z* (ESI) 251 $[M+H]^+$.

2-(2-Cyclohexyl-2-cyclopropylethyl)piperidine (6a)

Method D:[24] NH_4OAc (53 mg, 0.69 mmol), $NaBCNH_3$ (41 mg, 0.65 mmol) and 3 Å molecular sieves (100 mg) were added at 23 °C to a MeOH solution (2 mL) of dicarbonyl compound **5a** (78 mg, 0.31 mmol). The resulting mixture was stirred at 23 °C for 16 h before filtration through a pad of Celite®. The filtrate was concentrated under reduced pressure. The residue was purified by FC (silica gel, MeOH:CH_2Cl_2 0:100 → 10:90) to afford compound **6a** (58 mg, 78 %, mixture of diastereoisomers, purity > 97%) as a pale yellow oil. NMR spectra were recorded by using the HCl salt of the compound, which was prepared by treating **6a** (10 mg)

with HCl (200 µL, 1.25 M in MeOH) followed by concentration under reduced pressure. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.24 (brs, 2H), 3.51 – 3.38 (m, 1H), 3.27 (dd, *J* = 13.1, 7.0 Hz, 0.5H), 3.21 – 3.07 (m, 0.5H), 2.83 (dtd, *J* = 15.9, 12.7, 3.2 Hz, 1H), 2.22 – 1.53 (m, 12H), 1.53 – 0.96 (m, 7H), 0.85 (ddd, *J* = 16.2, 13.6, 6.8 Hz, 0.5H), 0.72 – 0.37 (m, 7.5H), 0.24 – 0.07 (m, 3H), 0.29 – 0.08 (m, 1.5H), 0.06 – -0.03 (m, 0.5 H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 55.9, 55.6, 44.8, 44.7, 44.5, 44.29, 42.9, 42.0, 36.40, 36.35, 30.9, 30.7, 30.0, 29.0, 28.4, 27.1, 26.94, 26.87, 26.75, 26.72, 22.5, 22.4, 22.3, 22.2, 13.61, 13.56, 4.9, 4.5, 4.0, 3.7. *m/z* (ESI) 236 $[M+H]^+$; HRMS (ESI) calcd for $C_{16}H_{30}N^+$ $[M+H]^+$ 236.2373, found 236.2369.

Ethyl 4-(methoxymethoxy)cyclohexanecarboxylate (20)

DIPEA (992 µL, 5.8 mmol) and MOMCl (330 µL, 4.35 mmol) were added to a CH_2Cl_2 (7 mL) solution of ethyl 4-hydroxycyclohexanecarboxylate (500 mg, 2.9 mmol) cooled to 0 °C. After stirring at r.t. for 6 h H_2O (5 mL) was added, the layers were separated and the organic phase was dried (Na_2SO_4). The solvent was evaporated and the crude was purified by FC (hexane:EtOAc 8:2) to give **20** (590 mg, 93% yield) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.47 (s, 1 H), 3.94 (q, 2 H, *J* = 7.0 Hz, 2 H), 3.58-3.51 (m, 1H), 3.17 (s, 3 H), 2.23 – 2.21 (m, 1 H), 1.93-1.78 (m, 2H), 1.76-1.65 (m, 2H), 1.63-1.52 (m, 2H), 1.51-1.41 (m, 3H), 1.07 (t, *J* = 7.0 Hz, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 175.0, 94.5, 74.7, 60.0, 54.9, 42.2, 29.6, 23.8, 14.0. *m/z* (ESI) 217 $[M+H^+]$

4-(Methoxymethoxy)cyclohexanecarbaldehyde (21)

NaOH (210 mg, 5.24 mmol) was added to a solution of **20** (570 mg, 2.62 mmol) in a THF:H_2O 4:1 mixture (10 mL). The reaction was stirred overnight, then H_2O (5 mL) was added. The layers were separated, the aqueous phase was acidified with 1*N* HCl until pH 1 was reached and then extracted with EtOAc (10 mL). The organic phase was dried (Na_2SO_4), filtered and the solvent was removed under reduced pressure to give the

corresponding acid (413 mg ,83% yield) as a white solid. Et_3N (250 µL, 1.80 mmol) and ethyl chloroformate (172 µL, 1.80 mmol) were added to a dry THF (5 mL) solution of acid (300 mg, 1.6 mmol) cooled to 0 °C. The resulting suspension was stirred at the same temperature for 1h and then filtered through Celite. The filtrate was then added to a solution of $NaBH_4$ (116 mg, 3.1 mmol) in water (2 mL) cooled to 0 °C. The reaction mixture was stirred at the same temperature for 30 min, quenched with H_2O (5 ml) and extracted with EtOAc (10 mL). The solvent was removed under reduced pressure and the crude was purified by FC (Hex: EtOAc 1:1) to give 298 mg of the corresponding alcohol (268 mg , 96% yield) as a colourless oil. A solution of $(COCl)_2$ (133 µL, 1.5 mmol) in CH_2Cl_2 (3 mL) was added dropwise to a solution of DMSO (204 µL, 2.87 mmol) in CH_2Cl_2 (5 mL), cooled to -78 °C. The reaction was stirred at the same temperature for 15 min, then a solution of the alcohol above (200 mg, 1.15 mmol) in CH_2Cl_2 (5 mL) was added. After stirring for 1 h, Et_3N (800 µL, 5.75 mmol) was added. After 30 min the reaction was warmed to r.t, quenched with 1*N* HCl (aq) and extracted with EtOAc (10 mL). The solvent was dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude was purified by FC (hexane:EtOAc 8:2) to give **58** (165 mg, 83%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.51 (s, 1 H), 4.52 (s, 2 H), 3.66-3.55 (m, 1H), 3.23 (s, 3 H), 2.20 – 2.23 (m, 1 H), 1.98-1.85 (m, 2H), 1.82-1.70 (m, 1H), 1.67-1.40 (m, 4H), 1.30 – 1.21 (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 204.2, 94.6, 74.6, 71.9, 55.1, 48.2, 31.1, 23.7, 21.2. *m/z* (ESI) 173 [$M+H^+$].

Cyclohexyl(4-(methoxymethoxy)cyclohexyl)methanol (22)

A small crystal of iodine was added to a suspension of magnesium shavings (63 mg, 2.58 mmol) in Et_2O (2 mL, nitrogen atmosphere). Bromocyclohexane (317 µL, 2.58 mmol) was then added slowly (over 5 min), so as to maintain a gentle reflux. The mixture was heated to reflux for 30 min. After cooling to r.t, aldehyde **21** (370 mg, 2.15 mmol) in Et_2O (2 mL,), was slowly added. After stirring at r.t overnight, NH_4Cl (5 mL, sat. aq.) was added and the reaction was extracted with EtOAc (10 mL). The organic layer was dried (Na_2SO_4), filtered

and concentrated under reduced pressure. The crude was purified by FC (hexane:EtOAc 7:3) to give **22** (233 mg, 91%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.59 (s, 2 H), 3.77 - 3.69 (m, 1H), 3.28 (s, 3H), 3.10-2.93 (m, 1H), 2.07- 1.92 (m, 1H), 1.88-1.77 (m, 1H), 1.74-1.73 (m, 3H), 1.62-1.26 (m, 8H), 1.25- 0.87 (m, 8H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 94.5, 79.7, 75.9, 71.3, 55.1, 40.2, 39.8, 39.0, 32.4, 30.2, 29.9, 28.0, 26.5, 24.0, 21.8. *m/z* (ESI) 257 [$M+H^+$]

Cyclohexyl(4-(methoxymethoxy)cyclohexyl)methanone (23)

PCC (846 mg, 2.25 mmol) was added to a solution of **22** (470 mg, 1.87 mmol) in CH_2Cl_2 (40 mL). The reaction was stirred overnight, then filtered through Celite. The filtrate was concentrated under reduced pressure and purified by FC (hexane:EtOAc 9:1) to give **23** (395 mg, 83%) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.56 (s, 2 H), 3.75 - 3.64 (m, 1H), 3.27 (s, 3H), 2.50-2.35 (m, 2H), 1.84-1.74 (m, 2H), 1.73-1.62 (m, 6H), 1.61-1.35 (m, 5H), 1.33- 1.05 (m, 5H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 216.4, 94.6, 71.0, 55.2, 48.7, 48.0, 29.8, 28.7, 25.8, 25.7, 23.1. *m/z* (ESI) 277.2 [$M+Na^+$]

4-(1-Cyclohexyl-2-(piperidin-2-yl)ethyl)cyclohexanol (24)

A solution of *n*BuLi (680 µL, 1.7 mmol 2.5 M in hexanes) was added dropwise to a stirred solution of 2-picoline (168 µL, 1.7 mmol) in THF (4 mL) at -78 °C. The resulting red solution was stirred at that temperature for 10 min before being warmed to -10 °C for 20 min, after which the mixture was cooled to -78 °C and a THF (2 mL) solution of **23** (395 mg, 1.55 mmol) was added. The resulting mixture was stirred at that temperature for 30 min before $SOCl_2$ was added (186 µL, 2.55 mmol). The milky reaction mixture was warmed to 0 °C for 30 min prior to quenching with NH_4Cl (10 mL, sat. aq.) and dilution with EtOAc (10 mL). The layers were separated and the organic layer was washed with brine (20 mL), dried (Na_2SO_4) and concentrated under reduced pressure. FC (hexane:EtOAc 8:2) gave the intermediate

unsaturated 2-pyridines (442 mg, 75%) as a yellow oil. To a solution of these 2-pyridines (210 mg, 0.63 mmol) in MeOH (7 mL) a catalytic amount of PtO_2 was added. The reaction was stirred under a hydrogen atmosphere for 24 h, filtered through a Celite® pad and concentrated under reduced pressure. The crude was purified by FC (CH_2Cl_2:MeOH 9:1) to give 2-(2-cyclohexyl-2-(4-(methoxymethoxy)cyclohexyl)ethyl)piperidine (180 mg, 84%) as a yellow oil.

TFA (120 µL, 1.55 mmol) was added to a CH_2Cl_2 (4 mL) solution of 2-(2-cyclohexyl-2-(4-(methoxymethoxy)cyclohexyl)ethyl)piperidine (175 mg 0.51 mmol). After stirring for 4 h the solvent was removed under reduced pressure and the crude dissolved in THF (3 mL). 1*N* aq. NaOH (1.5 mL) was added and the reaction was stirred for 1 h. H_2O (5 mL) and EtOAc (10 mL) were added and the layers were separated. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure to give **24** (130 mg, 86%, purity > 96%) as a yellow amorphous solid. .^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.93-3.83 (m, 1H), 2.98 (d, *J* = 12.1 Hz, 1H), 2.53 (td, *J* = 9.6 and 2.6 Hz, 1H), 2.39 – 2.27 (m, 1 H), 2.26 – 2.22 (m, 1 H), 1.68-0.81 (m, 30 H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 66.2, 57.0, 47.2, 44.5, 39.8, 39.4, 36.4, 33.1, 32.9, 32.9, 31.9, 29.6, 27.0, 26.8, 26.7, 26.3, 25.2, 24.9, 23.3. *m/z* (ESI) 294 [M+H$^+$]

In vitro CPT-1 Inhibition Assay:[29]

Preparation of Rat Heart Mitochondrial Suspension (MS): All of the steps below were performed on ice or at 4 °C. Three male Wistar rats (8 weeks of age) were sacrificed by cervical dislocation, hearts were excised, rinsed twice with ice-cold Homogenization Buffer (HB: 10 mM Tris-HCl, 250 mM sucrose, 1 mM EDTA, pH 7.4), blotted, weighed, and finely minced with scissors for 5 min. Minced hearts were homogenized in 5 volumes of HB (by tissue weight) using a Polytron homogenizer. The homogenate was centrifuged for 10 min at 600 x g, and the supernatant was removed and saved. The pellet was re-suspended in HB, homogenized again, spun for 10 min at 600 x g, and the supernatants from the first and second homogenizations were combined. The combined supernatant was centrifuged for 10

min at 7000 x g, the supernatant was discarded, the pellet was re-suspended in HB to the original homogenization volume and centrifuged again for 10 min at 7000 x g. The supernatant was discarded and the pellet of washed mitochondria was re-suspended in HB to 0.5 volumes relative to original heart weight. Protein content was determined using the Bradford protein assay, the solution was diluted with HB to a protein concentration of 10 mg/mL, and small individual aliquots of this mitochondrial suspension (MS) were shock-frozen in LN_2 and stored at -80 °C. Aliquots of MS were thawed and diluted with HB to 2 mg/ml protein just prior to use in CPT-1 assays.

Each reaction (final volume 200 µL, pH 6.8) contained 0.04 mg of MS protein, 25 mM Tris-HCl, 150 mM sucrose, 1 mM EDTA, 60 mM KCl, 1.2 mg/mL lipid-free BSA, 0.1 mM 4,4'-dithiodipyridine, 0.4 mM L-carnitine, 0.04 mM palmitoyl CoA and varying concentrations of inhibitor. Reactions were performed in a quartz glass microcuvette, and inhibitors were pre-incubated for 3 min at 37 °C with all assay components except for L-carnitine and palmitoyl CoA. Each inhibitor was dried down from a CH_2Cl_2 stock, redissolved in the assay buffer described above, and appropriate serial dilutions were made. A solution of L-carnitine and palmitoyl CoA (in water) was added to initiate the enzyme reaction, and the cuvette was placed in a spectrophotometer programmed in kinetic mode to read the increase in absorbance at 324 nm for 3.5 min. The slopes of the kinetic curves were calculated and compared to the mean of positive controls generated in the absence of any inhibitor (defined as 100 % activity), to determine the % control activity for each inhibitor concentration. Controls and each inhibitor concentration were analyzed with 5 replicates, and mean +/- SD were calculated for each point. IC_{50} estimates and 95% confidence intervals for the malonyl CoA-inhibitable component of the CPT activity were determined following subtraction of the non-inhibited activity by non-linear regression of inhibition curves using GraphPad Prism 5 (GraphPad Software Inc), and each value listed in Table 2 represents the mean IC_{50} value and pooled confidence interval from two independent experiments using each inhibitor.

***Ex vivo* Langendorff Model:**

Briefly, C57BL/6 (25 - 30 g) murine hearts were isolated and mounted onto the Langendorff perfusion system and retrogradely perfused with a modified Krebs buffer consisting of 1.2 mM sodium palmitate pre-conjugated to 3% bovine serum albumin (BSA).[36] The buffer was perfused at a constant pressure of 80 mmHg at 37 °C and continuously gassed with 95% O_2 / 5% CO_2. Following 10 min stabilisation in control (BSA-Palmitate) Krebs buffer, hearts were perfused for 30 min with 1) control (BSA-Palmitate), 2) 2 μM perhexiline, 3) 2 μM **50,** or 4) 10 μM **50**. The maximal and minimal change in left ventricular pressure over time (LVdP/dt max and min, respectively) were calculated from the left ventricular pressure recorded throughout the 40 minutes of perfusion. All data are expressed as mean ± SEM and statistical comparison between groups were performed using 1-way ANOVA, followed by the post-hoc Bonferroni test. Values of $P<0.05$ were deemed as significant.

Pharmacokinetics assays:

Routine in vitro stability studies were conducted by Cyprotex Ltd (Macclesfield, UK) and in vivo pharmacokinetics were conducted by Sai Life (Pune, India) using standard methodology.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXXXX. Experimental procedures, characterization of all intermediates and target compounds, and copies of NMR spectra of compounds **6a-j**, **31**, **42**, **50**, **55**, **62**.

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Notes:

M.P.F., M.Z. and I.R.G. are co-founders of Signal Pharma Limited that owns the I.P. of some of the molecules described in this article.

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ABBREVIATIONS USED

CYP2D6, cytochrome P450 2D6; CPT-1, carnitine palmitoyltransferase-1; CPT-2, carnitine palmitoyltransferase-2; HF, heart failure; DMP, Dess-Martin periodinane; HLM, human liver microsomes; RLM, rat liver microsomes; LVdP, Left Ventricular developed Pressure; SEM, Standard Error of Mean.

REFERENCES

(1) Hudak, W. J.; Lewis, R. E.; Kuhn, W. L. Cardiovascular Pharmacology of Perhexiline. *J. Pharmacol. Exp. Ther.* **1970**, *173*, 371–382.

(2) Cho, Y. W.; Belej, M.; Aviado, D. M. Pharmacology of a New Antianginal Drug: Perhexiline : I. Coronary Circulation and Myocardial Metabolism. *Chest* **1970**, *58*, 577–581.

(3) Singlas, E.; Goujet, M. A.; Simon, P. Pharmacokinetics of Perhexiline Maleate in Anginal Patients with and without Peripheral Neuropathy. *Eur. J. Clin. Pharmacol.* **1978**, *14*, 195–201.

(4) Lee, L.; Campbell, R.; Scheuermann-Freestone, M.; Taylor, R.; Gunaruwan, P.; Williams, L.; Ashrafian, H.; Horowitz, J.; Fraser, A. G.; Clarke, K.; Frenneaux, M.

Metabolic Modulation With Perhexiline in Chronic Heart Failure. *Circulation* **2005**, *112*, 3280–3288.

(5) Willoughby, S. R.; Stewart, S.; Chirkov, Y. Y.; Kennedy, J. A.; Holmes, A. S.; Horowitz, J. D. Beneficial Clinical Effects of Perhexiline in Patients with Stable Angina Pectoris and Acute Coronary Syndromes Are Associated with Potentiation of Platelet Responsiveness to Nitric Oxide. *Eur. Heart J.* **2002**, *23*, 1946–1954.

(6) Unger, S. A.; Robinson, M. A.; Horowitz, J. D. Perhexiline Improves Symptomatic Status in Elderly Patients with Severe Aortic Stenosis. *Aust. N. Z. J. Med.* **1997**, *27*, 24–28.

(7) Abozguia, K.; Elliott, P.; McKenna, W.; Phan, T. T.; Nallur-Shivu, G.; Ahmed, I.; Maher, A. R.; Kaur, K.; Taylor, J.; Henning, A.; Ashrafian, H.; Watkins, H.; Frenneaux, M. Metabolic Modulator Perhexiline Corrects Energy Deficiency and Improves Exercise Capacity in Symptomatic Hypertrophic CardiomyopathyClinical Perspective. *Circulation* **2010**, *122*, 1562–1569.

(8) Galluzzi, L.; Kepp, O.; Heiden, M. G. V.; Kroemer, G. Metabolic Targets for Cancer Therapy. *Nat. Rev. Drug Discovery* **2013**, *12*, 829–846.

(9) Vander Heiden, M. G. Targeting Cancer Metabolism: A Therapeutic Window Opens. *Nat. Rev. Drug Discovery* **2011**, *10*, 671–684.

(10) Tseng, C.-C.; Greig, I.; Harrison, W.; Zanda, M. Asymmetric Synthesis and Absolute Configuration of (+)- and (–)-Perhexiline. *Synthesis* **2015**, *48*, 73–78.

(11) Ashrafian, H.; Horowitz, J. D.; Frenneaux, M. P. Perhexiline. *Cardiovasc. Drug Rev.* **2007**, *25*, 76–97.

(12) Licari, G.; Somogyi, A. A.; Milne, R. W.; Sallustio, B. C. Comparison of CYP2D Metabolism and Hepatotoxicity of the Myocardial Metabolic Agent Perhexiline in Sprague-Dawley and Dark Agouti Rats. *Xenobiotica* **2015**, *45*, 3–9.

(13) Chong, C-R.; Drury, N. L.; Licari, G.; Frenneaux, M. P.; Horowitz, J. D.; Pagano, D.; Sallustio, B. C. Stereoselective handling of perhexiline: implications regarding

accumulation within the human myocardium. *Eur. J. Clin. Pharmacol.* **2015**, *71*, 1485–1491.

(14) Sallustio, B.; Milne, R.; Licari, J.; Somogyi, A. A. Uses of (-)-Perhexiline. WO2014036603 (A1), March 13, 2014.

(15) Davies, B. J.; Coller, J. K.; Somogyi, A. A.; Milne, R. W.; Sallustio, B. C. CYP2B6, CYP2D6, and CYP3A4 Catalyze the Primary Oxidative Metabolism of Perhexiline Enantiomers by Human Liver Microsomes. *Drug Metab. Dispos.* **2007**, *35*, 128–138.

(16) George, C. H.; Mitchell, A. N.; Preece, R.; Bannister, M. L.; Yousef, Z. Pleiotropic Mechanisms of Action of Perhexiline in Heart Failure. *Expert Opin. Ther. Pat.* **2016**, *26*, 1049–1059.

(17) Kennedy, J. A.; Unger, S. A.; Horowitz, J. D. Inhibition of Carnitine Palmitoyltransferase-1 in Rat Heart and Liver by Perhexiline and Amiodarone. *Biochem. Pharmacol.* **1996**, *52*, 273–280.

(18) Ceccarelli, S. M.; Chomienne, O.; Gubler, M.; Arduini, A. Carnitine Palmitoyltransferase (CPT) Modulators: A Medicinal Chemistry Perspective on 35 Years of Research. *J. Med. Chem.* **2011**, *54*, 3109–3152.

(19) Leclerc, G.; Decker, N.; Schwartz, J. Synthesis and Cardiovascular Activity of a New Series of Cyclohexylaralkylamine Derivatives Related to Perhexiline. *J. Med. Chem.* **1982**, *25*, 709–714.

(20) Tassoni, E.; Giannessi, F.; Dell', U. N.; Gallo, G.; Conti, R.; Tinti, M. O. Inhibitors of CPT in the Central Nervous System as Antidiabetic and/or Anti-Obesity Drugs. WO2007096251 A1, August 30, 2007.

(21) Tilford, C. H.; Van Campen, M. G. Diuretics. α,α-Disubstituted 2-Piperidine-Ethanols and 3,3-Disubstituted Octahydropyrid[1,2-c]oxazines. *J. Am. Chem. Soc.* **1954**, *76*, 2431–2441.

(22) Schou, S. C. Influence of [^{2}H]-Labelled Acetic Acid as Solvent in the Synthesis of [^{2}H]-Labelled Perhexiline. *J. Labelled Compd. Radiopharm.* **2010**, *53*, 31–35.

(23) Lipshutz, B. H.; Wilhelm, R. S.; Kozlowski, J. A. Conjugate Addition Reactions of α,β-Unsaturated Ketones with Higher Order, Mixed Organocuprate Reagents, $R_2Cu(CN)Li_2$. *J. Org. Chem.* **1984**, *49*, 3938–3942.

(24) Saavedra, O. M.; Martin, O. R. Chemical Synthesis of β-Homonojirimycin, of Its N-Butyl Derivative, and of "Methyl Homoazacellobioside." *J. Org. Chem.* **1996**, *61*, 6987–6993.

(25) Gillis, E. P.; Eastman, K. J.; Hill, M. D.; Donnelly, D. J.; Meanwell, N. A. Applications of Fluorine in Medicinal Chemistry. *J. Med. Chem.* **2015**, *58*, 8315–8359.

(26) Furuya, T.; Strom, A. E.; Ritter, T. Silver-Mediated Fluorination of Functionalized Aryl Stannanes. *J. Am. Chem. Soc.* **2009**, *131*, 1662–1663.

(27) Inglis, S. C.; Herbert, M. K.; Davies, B. J. L.; Coller, J. K.; James, H. M.; Horowitz, J. D.; Morris, R. G.; Milne, R. W.; Somogyi, A. A.; Sallustio, B. C. Effect of CYP2D6 Metabolizer Status on the Disposition of the (+) and (-) Enantiomers of Perhexiline in Patients with Myocardial Ischaemia. *Pharmacogenet. Genomics* **2007**, *17*, 305–312.

(28) Davies, B. J.; Herbert, M. K.; Coller, J. K.; Somogyi, A. A.; Milne, R. W.; Sallustio, B. C. Determination of the 4-Monohydroxy Metabolites of Perhexiline in Human Plasma, Urine and Liver Microsomes by Liquid Chromatography. *J. Chromatogr. B: Biomed. Sci. Appl.* **2006**, *843*, 302–309.

(29) McGarry, J. D.; Leatherman, G. F.; Foster, D. W. Carnitine palmitoyltransferase I. The site of inhibition of hepatic fatty acid oxidation by malonyl-CoA. *J. Biol. Chem.* **1978**, *253*, 4128–4136.

(30) Doenst, T.; Nguyen, T. D.; Abel, E. D. Cardiac Metabolism in Heart Failure. *Circ. Res.* **2013**, *113*, 709–724.

(31) Aasum, E.; Hafstad, A. D.; Larsen, T. S. Change in Substrate Metabolism in Isolated Mouse Hearts Following Ischemia-Reperfusion. *Mol. Cell. Biochem.* **2003**, *249*, 97–103.

(32) Borlaug, B. A.; Paulus, W. J. Heart Failure with Preserved Ejection Fraction: Pathophysiology, Diagnosis, and Treatment. *Eur. Heart J.* **2011**, *32*, 670–679.

(33) Gutierrez, C.; Blanchard, D. G. Diastolic Heart Failure: Challenges of Diagnosis and Treatment. *Am. Fam. Physician* **2004**, *69*, 2609–2616.

(34) Loudon, B. L.; Noordali, H.; Gollop, N. D.; Frenneaux, M. P.; Madhani, M. Present and Future Pharmacotherapeutic Agents in Heart Failure: An Evolving Paradigm. *Br. J. Pharmacol.* **2016**, *173*, 1911–1924.

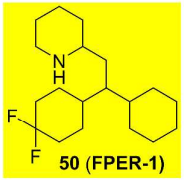
(35) Still, W. C.; Kahn, M.; Mitra, A. Rapid Chromatographic Technique for Preparative Separations with Moderate Resolution. *J. Org. Chem.* **1978**, *43*, 2923–2925.

(36) Kennedy, J. A.; Kiosoglous, A. J.; Murphy, G. A.; Pelle, M. A.; Horowitz, J. D. Effect of perhexiline and oxfenicine on myocardial function and metabolism during low-flow ischemia/reperfusion in the isolated rat heart. *J. Cardiovasc. Pharmacol.* **2000**, *36*, 794–801

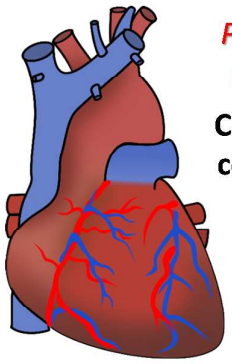
TOC

- Significantly increased *in vitro* metabolic stability
- Good *in vitro* potency
- Selective concentration in the myocardium
- Improvements in cardiac contractility and relaxation

Perhexiline (PHX)



50 (FPER-1)



Perhexiline and FPER-1 effect

Carbohydrate consumption ↑

Fatty acid consumption ↓

Myocardial efficiency ↑