Novel 2-arylthiopropanoyl-CoA inhibitors of α-methylacyl-CoA racemase 1A (AMACR; P504S) as potential anti-prostate cancer agents

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ABSTRACT

α-Methylacyl-CoA racemase (AMACR; P504S) catalyses an essential step in the degradation of branched-chain fatty acids and the activation of ibuprofen and related drugs. AMACR has gained much attention as a drug target and biomarker, since it is found at elevated levels in prostate cancer and several other cancers. Herein, we report the synthesis of 2-(phenylthio)propanoyl-CoA derivatives which provided potent AMACR inhibitory activity (IC_{50} = 22–100 nM), as measured by the AMACR colorimetric activity assay. Inhibitor potency positively correlates with calculated logP, although 2-(3-benzyloxyphenylthio)propanoyl-CoA and 2-(4-(2-methylpropoxy)phenylthio)propanoyl-CoA were more potent than predicted by this parameter. Subsequently, carboxylic acid precursors were evaluated against androgen-dependent LnCaP prostate cancer cells and androgen-independent Du145 and PC3 prostate cancer cells using the MTS assay. All tested precursor acids showed inhibitory activity against LnCaP, Du145 and PC3 cells at 500 μM, but lacked activity at 100 μM. This is the first extensive structure-activity relationship study on the influence of side-chain interactions on the potency of novel rationally designed AMACR inhibitors.

1. Introduction

Branched-chain fatty acids, *e.g.* phytanic and pristanic acids, are common components of the human diet [1–3]. Degradation of these fatty acids occurs as the corresponding acyl-CoA, initially within peroxisomes and subsequently within mitochondria [3,4]. Phytanic acid possesses a 3-methyl group, which hinders degradation by β-oxidation [1–4]. Therefore, initial metabolism is via the α-oxidation pathway, resulting in the formation of pristanic acid, which possesses a 2-methyl group, which is subsequently converted into pristanoyl-CoA. The stereochemical configuration of chiral centres in phytanic acid are retained in the α-oxidation pathway [3] and this means that *R*-2-

methylacyl-CoAs are generated. Oxidation of cholesterol to bile acids also results in the formation of *R*-2-methylacyl-CoAs (with the chiral centre at carbon-25 in the standard numbering system for bile acids) [1,2]. The acyl-CoA oxidases catalysing the first step in the β-oxidation degradative pathway requires *S*-2-methylacyl-CoAs [5–7] and cannot accept *R*-2-methylacyl-CoAs. This means that 2*R*-pristanoyl-CoA, its β-oxidation pathway products and the acyl-CoA derivatives of bile acids cannot be directly metabolized by β-oxidation.

The enzyme α-methylacyl-CoA racemase (AMACR; P504S; E.C. 5.1.99.4) catalyses conversion of these *R*-2-methylacyl-CoAs to *S*-2-methylacyl-CoAs, thus enabling β-oxidation. The reaction appears to occur by a deprotonation/reprotonation mechanism [8–10], probably

through an enolate intermediate [11], and a near 1:1 mixture of *S*- and *R*-2-methylacyl-CoA epimers is produced ('racemization') [9,10,12]. The resulting *S*-2-methylacyl-CoAs are degraded by β-oxidation [1–4], while the resulting *R*-2-methylacyl-CoAs are further metabolized to their *S*-epimers by AMACR, thus ensuring complete metabolism. In addition to its role in fatty acid metabolism, AMACR is also involved in the irreversible pathway which converts *R*-ibuprofen to *S*-ibuprofen via the corresponding acyl-CoA esters [1,2,10]. As only the *S*-enantiomer of ibuprofen is a potent inhibitor of cyclooxygenases-1 and -2 [13], this pathway results in the pharmacological activation of the inactive *R*-ibuprofen. Several other 'profens' also undergo similar *R*- to *S*-conversion in humans and other mammals [1,2,10]. AMACR has also been claimed to be involved in the *S*- to *R*- conversion of mandelic acid in mammalian cells [14], but this role was subsequently disproved [15].

There has been increased attention towards targeting AMACR as a chemotherapeutic strategy to combat prostate cancer (PCa). AMACR activity is increased by 4- to 10-fold in clinical prostate cancer tissue samples, compared with the corresponding normal tissue [16,17]. Many spliced variants of AMACR have been reported [18–21], with the most highly expressed spliced variant, AMACR 1A, demonstrated to have 'racemase' activity [9,12]. Several other spliced variants of AMACR are predicted not to possess racemase activity, as they lack key catalytic residues or a C-terminal dimerization domain [1,2,18–21]. Knockdown of AMACR 1A significantly reduces the proliferation of several androgen-dependent (AD) [17,22] and androgen-independent (AI) [23] PCa cell lines at similar levels to that observed for the current standard of care (androgen-deprivation) or androgen-receptor (AR) antagonists (Casodex) [17]. Additionally, knock-down of AMACR has shown to revert advanced PCa cells from AI to AD status by post-transcriptional upregulation of the AR [23]. Many investigations, *e.g.* Takahara *et al.* [23], demonstrate that AMACR is over-expressed at significantly higher levels in AI PCa cells compared to AD PCa cells, which has stimulated great interest in exploring AMACR as a PCa biomarker [1,24–27] and drug target [22,24,28–33]. Inhibitors of AMACR could be exploited as mono-therapeutic agents or used in combination with androgen-deprivation therapy to target CRPC [17,23].

Most previously reported AMACR inhibitors have been developed using rational design, based on substrate structures and the enzyme catalytic mechanism [24,28–30,33]. Inhibitors of the *M. tuberculosis* homologue enzyme (*Mt*MCR), for which X-ray crystal structures have been solved [8,11,34], include the *gem*-disubstituted substrate-product analogues 2,2-bis(4-(2-methylpropyl)lphenyl)propanoyl-CoA and 2,2-bis(4-tert-butylphenyl)propanoyl-CoA, which inhibit *Mt*MCR with K_i values of 16.9 ± 0.6 and 21 ± 4 μM, respectively [32]. Carbamate analogues of these inhibitors have recently been reported to be irreversible inhibitors of *Mt*MCR [31]. However, until recently, there has been no convenient assay to measure AMACR activity or evaluate the potency of inhibitors, and this has hampered inhibitor development [35,36]. We recently developed a continuous assay which is convenient to use for inhibitor characterization, using a chromogenic substrate which produces the intensely yellow 2,4-dinitrophenolate anion upon incubation with AMACR [30,36].

Herein, we report the synthesis of a panel of novel 2-arylthiopropanoyl-CoA derivatives ('thiolactate inhibitors'). These inhibitors were tested *in vitro* against human AMACR 1A in the first systematic examination of rational inhibitor side-chain interactions and their influence on inhibitor potency. We then examined selected examples of the carboxylic acid precursors of these inhibitors against a panel of AD- and AI-PCa cell lines to evaluate their potential as treatments for prostate cancer.

2. Results and discussion

2.1. Rational design of inhibitors

The X-ray crystal structure of mammalian AMACR has not been

reported to date; however, numerous structures of ligands bound to the *M. tuberculosis* homologue (*Mt*MCR) [8,11] reveal the binding mode of several known substrates/inhibitors. There is 43% sequence identity between *Mt*MCR and AMACR 1A [22] (Supplementary Information, Fig. S1), including residues comprising active site catalytic bases, residues binding to the CoA moiety, and other active site residues. Therefore, structural information can be used to help design rational inhibitors of AMACR.

These *Mt*MCR structures suggest that AMACR 1A contains a well-defined binding site for the CoA moiety, which is lined by cationic arginine and lysine residues binding to the phosphate groups. The methyl group is thought to bind in a well-defined pocket, and this hypothesis is supported by the observation that the presence of an α-methyl group [30] (or similar group [29]) enhances inhibition of AMACR, compared to corresponding analogues that contain a hydrogen atom at the same position. Similarly, biochemical studies show that 2-methylacyl-CoA substrates are better substrates than those lacking the α-methyl group [37]. The α-carbon of the 2-methylacyl-CoA substrate is positioned so that the active site bases (His-122/Glu-241 and Asp-152; numbering refers to human AMACR 1A) are located either side of the α-carbon in order to perform the deprotonation/reprotonation reaction effectively. Two overlapping side-chain binding sites, termed the *R*- and *S*-pockets, accommodate substrate/inhibitor side-chains and this probably accounts for the observed differences in potency between different epimers of 2-methylacyl-CoA-like inhibitors [28]. This led to the design of gem-disubstituted substrate-product analogues, which are thought to inhibit *Mt*MCR by simultaneous binding of side-chains to both the *R*- and *S*-binding sites [31].

Previous work on AMACR have attempted to develop inhibitors by increasing the acidity of the α-proton [28] or by mimicking the planar enolate intermediate [24,29,30,36]. The first inhibitors with highly acidic α-protons contained fluorine [28] but these were subsequently shown to be substrates which eliminate HF and form the corresponding unsaturated analogue [12,30,35]. We therefore decided to prepare a focused set of 2-arylthiopropanoyl-CoA analogues ('thiolactate inhibitors'), in the expectation that these would form stabilized enolates which would bind tightly to AMACR. Previous work on 3-thia analogues of straight-chain acyl-CoA oxidase substrates confirm a diminution of the pK_a of the α-proton by *ca.* 5 units [38–41] (~16 for 3-thiaoctanoyl-CoA *vs.* ~ 21 for octanoyl-CoA [38]), supporting this approach.

The target compounds were synthesized as epimeric mixtures at the 2-position, as formation of the enolate and loss of stereochemical configuration was anticipated to be facile. In parallel, we also investigated the corresponding sulfone analogues, reasoning that the sulfone group would promote formation of the enolate even more strongly, potentially resulting in even more potent inhibition. Fenoprofenoyl-CoA **1** provided the starting side-chain structure, as this was previously shown to be the best of the series of substrates with aromatic side-chains [10] and is a known inhibitor [30].

2.2. Synthesis of inhibitors

Synthesis of initially designed inhibitors used the reaction of 3- or 4-phenoxybenzenethiol **1** with ethyl (±)-2-bromopropanoate to afford the ethyl esters **2** (Scheme 1). The required phenoxybenzenethiols **1** have been synthesized from the corresponding phenoxyphenols **3** by treatment with dimethylthiocarbamoyl chloride [42] to give **4**. A Newman-Kwart thermal rearrangement was subsequently used to provide the required carbamothioate **5**. Only low levels of conversion were observed at 260 °C [43], while extensive decomposition occurred at 300 °C. A compromise temperature of 280 °C gave the required product in moderate yield whilst minimizing decomposition. Hydrolysis of **5** furnished the required phenoxybenzenethiols **1** but these rapidly oxidized to the corresponding disulfides so they were treated with ethyl ± 2-bromopropanoate without purification. The resulting esters **2** were hydrolyzed to the acids **6**, which were activated with N,N′-
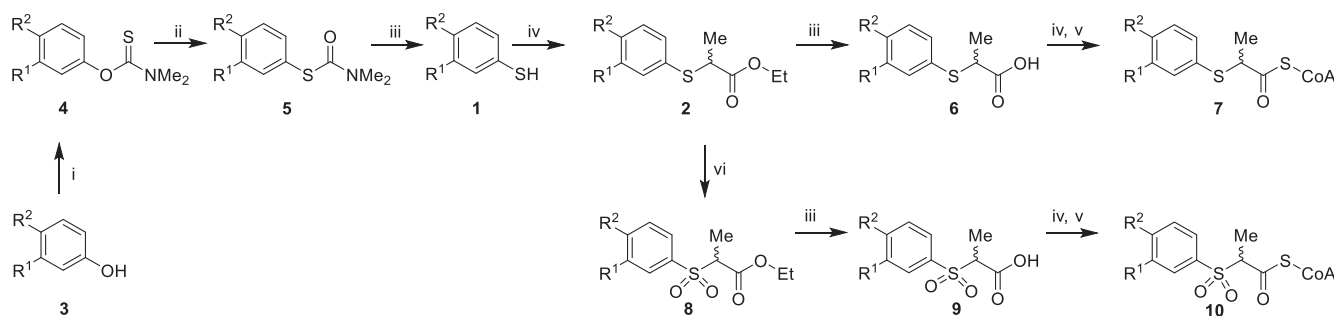
Scheme 1. Synthesis of 2-arylthiopropanoyl-CoAs **7** and 2-arylsulfonylpropanoyl-CoAs **10**. **a**: R^1 = PhO, R^2 = H; **b**: R^1 = H, R^2 = PhO. Reagents and conditions: i. dimethylthiocarbamoyl chloride, DMF, ii. Δ; iii. NaOH, H_2O, MeOH; iv. K_2CO_3, ethyl (±)-2-bromopropanoate, MeCN; iv. CDI, CH_2Cl_2; v. CoA-Li_3^+, $NaHCO_3$, H_2O, THF; vi. OXONE®, H_2O. Note that no product was obtained for **10a**.

carbonyl diimidazole and converted into their corresponding acyl-CoA esters **7** [10,12,30,36,44].

Synthesis of the sulfone analogues was achieved by oxidation of the ethyl ester intermediate **2** (Scheme 1). OXONE® treatment [45] of **2** resulted in formation of the sulfone ethyl esters **8** in *ca.* 80% yield, which were subsequently hydrolyzed to the acids **9** with aqueous base in high yield. Conversion of the 3-substituted-acid **9a** to the corresponding acyl-CoA **10a** was attempted using the standard carbonyl diimidazole method [10,12,30,36,44] but this did not result in the desired product. This failure may be due to the low nucleophilicity of the carboxylate anion of **9a**, as further experiments showed low yields of the intermediate acyl-imidazole. Alternatively, formation of the enolate may be facile under the conditions used to make the acyl-CoA and this results in side-reactions or depletion of base resulting in low levels of the required imidazole intermediate. Similarly, use of a mixed anhydride method [9,24] did not give **10a** and starting material was recovered. Activation of the isomeric carboxylic acid **9b** with carbonyl diimidazole and coupling with reduced CoA [10,12,30,36,44] resulted in formation of the corresponding acyl-CoA ester **10b** in low yield.

Preparation of further 2-arylthiopropanoyl-CoA derivatives **7c-o** (Scheme 2) began with selective alkylation of a mercaptophenol **11** with ethyl (±)-2-bromopropanoate to provide the corresponding ethyl (±)-2-(hydroxyphenylthio)propanoates **12**. Alkylation of the phenolic oxygen of **12** with benzyl or alkyl bromide derivatives provided a series of substituted benzyloxy or alkoxy esters **2c-o**. Hydrolyses of the esters revealed the corresponding carboxylic acids **6c-o**, which were converted into their CoA esters **7c-o** using N,N′-carbonyl diimidazole.

2.3. Evaluation of compounds in vitro

Initially compounds **7a** and **7b** were tested using our deuterium-exchange assay [9,10] (Supporting information, Fig. S2). Significant exchange of **7a/7b** α-^{1}H for α-^{2}H was observed in negative controls containing heat-inactivated AMACR, showing that formation of the enolate occurred rapidly. In the case of the 3-substituted isomer, **7a** *ca.* 70% exchange was observed in the absence of active AMACR. Incubation in the presence of active AMACR resulted in even greater levels of exchange for the 4-substituted isomer **7b**, indicating that this compound was a substrate. In the case of the 3-substituted isomer **7a**, limited further exchange was observed against the high non-enzymatic background levels showing that it was also a substrate.

Inhibition of AMACR activity was subsequently evaluated using our colorimetric assay [30,36]. Initial evaluation showed that **7a** and **7b** were moderately potent inhibitors, with IC_{50} values of 247 nM and 354 nM, respectively. Fenoprofenoyl-CoA **1** showed an IC_{50} value of 340 nM (consistent with the literature report of 400 nM [30]). Thus, both phenoxy-phenylthiopropanoyl-CoAs **7a** and **7b** were inhibitors of comparable potency to fenoprofenoyl-CoA **1**. In contrast, the 4-phenoxyphenylsulfone analogue **10b** showed an IC_{50} value of *ca.* 46 μM, showing a highly significant decrease in potency compared to **1**. This
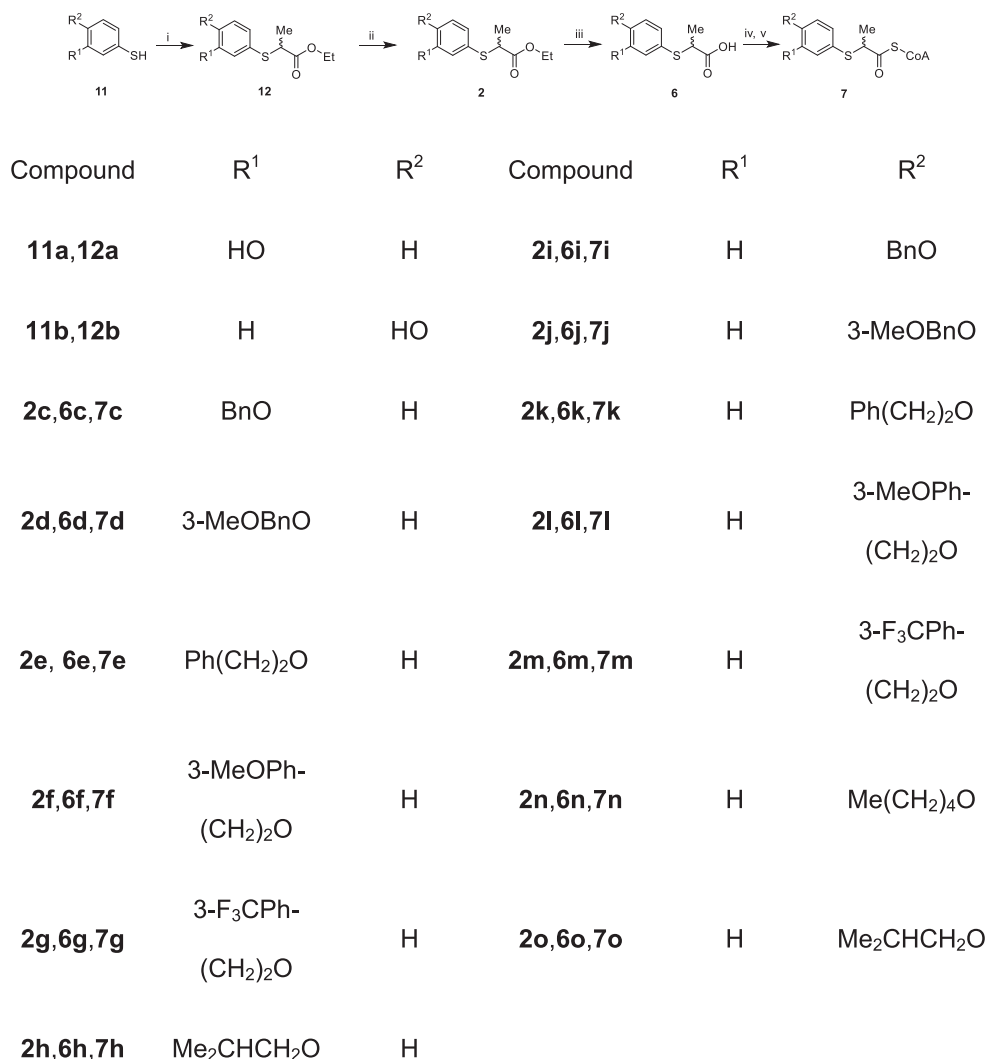
diminution in inhibitory activity was unexpected, as **10b** was expected to undergo exchange of the α-proton even more readily than the thia analogues and hence was predicted to bind to AMACR even more tightly. This loss of inhibitory activity correlates with a reduction in lipophilicity [46] (Table 1, Fig. 1, Supporting Information Fig. S4), suggesting that potency is largely driven by side-chain interactions rather than formation of the enolate. Further experiments showed that all three inhibitors gave rise to rapidly reversible inhibition (Supporting Information).

The further series of 3- and 4-substituted 2-arylthiopropanoyl-CoAs **7c-o** were then tested as inhibitors of AMACR activity (Table 1). As expected, potency was generally correlated with drug (side-chain) lipophilicity, although there were exceptions (Fig. 1 and Supporting Information Figs. S3 and S4). This is consistent with the proposed model in which the inhibitor side-chain binds to the methionine-rich surface ('side-chain binding site'), as observed in the X-ray crystal structures [8] of ligands bound to the *M. tuberculosis* homologue, MCR.

In the 3-substituted series, the most potent inhibition was observed for the benzyl derivative **7c** (IC_{50} = 22.3 nM) and this was far more potent than expected based on calculated lipophilicity (Fig. S3). Addition of a 3-MeO group to the benzyl to give **7d** decreased potency to the level predicted by the lipophilicity. Extending the side-chain with a further CH_2- group in **7e** did not make a significant difference in potency, compared to **7d**. Addition of a MeO- group to the terminal aromatic ring in **7f** gave a small improvement in potency. However, the expected increase in potency upon addition of a lipophilic trifluoromethyl- group was not observed and **7g** had a similar level of potency as **7f** (Fig. S3). Compound **7h**, substituted with a 3-(2-methylpropoxy) group, was less potent, as expected based on lipophilicity calculations.

In contrast, substitutions at the 4-position gave potent inhibition for almost all derivatives. Extension of the linker from **7b** (no CH_2- groups) to **7i** (one CH_2- group) resulted in an increase in potency (IC_{50} = 354 nM *vs.* 113 nM). Addition of a MeO-group to the terminal aromatic ring in **7j** made a small difference to the potency (IC_{50} = 71 nM *vs.* 113 nM]. Analogue **7k** had similar potency [IC_{50} = 99 nM *vs.* 113 nM] to **7i**, showing that increasing the linker by a further CH_2 group did not have a significant effect, as observed for the corresponding regioisomers. Substitution of the terminal phenyl ring with a MeO- group in **7l** or with a CF_3 group in **7m** gave similar levels of inhibitory potency, again showing that inhibitor potency was not increased as much as expected upon introduction of the lipophilic trifluoromethyl- group. 4-Substitution with an aliphatic pentoxyl chain also resulted in potent inhibition, as shown by **7n** with an IC_{50} value of 90 nM, consistent with predictions based on lipophilicity. Surprisingly, substitution with the smaller 2-methylpropoxy- group in **7o** gave five-fold more potent inhibition than in the 3-substituted analogue **7h** (IC_{50} = 106 nM *vs.* 522 nM).

As expected, activity in the presence of inhibitors was reversed upon rapid dilution, showing reversible inhibition for all compounds

Compound	R^1	R^2	Compound	R^1	R^2
11a,12a	HO	H	**2i,6i,7i**	H	BnO
11b,12b	H	HO	**2j,6j,7j**	H	3-MeOBnO
2c,6c,7c	BnO	H	**2k,6k,7k**	H	$Ph(CH_2)_2O$
2d,6d,7d	3-MeOBnO	H	**2l,6l,7l**	H	3-MeOPh-$(CH_2)_2O$
2e, 6e,7e	$Ph(CH_2)_2O$	H	**2m,6m,7m**	H	3-F_3CPh-$(CH_2)_2O$
2f,6f,7f	3-MeOPh-$(CH_2)_2O$	H	**2n,6n,7n**	H	$Me(CH_2)_4O$
2g,6g,7g	3-F_3CPh-$(CH_2)_2O$	H	**2o,6o,7o**	H	Me_2CHCH_2O
2h,6h,7h	Me_2CHCH_2O	H			

Scheme 2. Synthesis of further 2-arylthiopropanoyl-CoAs **7c-o** designed as inhibitors of AMACR. Reagents and conditions: i, ethyl (±)-2-bromopropanoate, $CHCl_3$, NEt_3; ii, R-Br, DMF, K_2CO_3 or R-OH, THF, PPh_3, DIAD; iii, NaOH, H_2O, MeOH; iv, CDI, CH_2Cl_2; v CoA-Li_3^+, $NaHCO_3$, H_2O, THF.

(Supporting information). Kinetic analysis using varying substrate concentrations in the presence or absence of 22.5 nM **7c** was used to determine the mode of inhibition. This analysis showed that mixed competitive inhibition was the most likely kinetic mode of action, with a K_i value of 14.6 ± 2.0 nM (mean ± S.E.M.) (Supporting information).

2.4. Cellular evaluation of compounds

Inhibitors of AMACR have been evaluated as their carboxylic acid precursors at the cellular level because of the poor cell permeability, the hydrolytic instability of the acyl-CoAs, and the known facile acyl-CoA formation from carboxylate inhibitor precursors in cells [24,28]. Therefore, the carboxylic acid precursors (**6c,h-j,n,o**) were tested against AD (LnCaP) and AI (Du145 and PC3) prostate cancer cell lines. The selected compounds were chosen as the corresponding acyl-CoA esters represented a range of 3- and 4-substituted derivatives (**7c,h-j,n,o**) with the inhibitors giving a range of activities, including the most potent (**7c**). Fenoprofen was included as a positive control.

These compounds were initially screened at 500 µM, 200 µM and 100 µM against the three cell lines. The 2-arylthiopropanoic acids **6** provided strong anti-proliferative activity (33–88%; Supporting Information, Table S1) against all three prostate cancer cell lines at 500 µM, and moderate activity at 200 µM. However, no significant
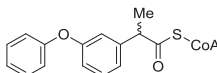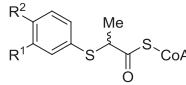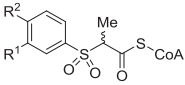
activity was observed at 100 µM. 'Unusual' fatty acids and derivatives are generally taken up into cells with subsequent import into peroxisomes as their acyl-CoA esters for metabolism and detoxification [1,4]; it was therefore anticipated that **6** would be taken up into cells (as has been shown for other AMACR inhibitor precursors [24]) and co-localize with AMACR in peroxisomes and mitochondria [47,48]. Conversion of the carboxylic acid into the acyl-CoA ester to produce the drug was expected to take place. It appears that **6** are not converted efficiently into **7**, and that this is responsible for the observed lack of activity. It is also possible that inhibitors **7** are removed by rapid metabolism, but previous work on 3-thia acyl-CoAs have suggested this is not the case [38,41,49].

3. Conclusions

Development of the 2-arylthiapropanoyl-CoA ('thiolactate') series of inhibitors demonstrates that highly potent inhibition of AMACR can be achieved *in vitro*. The structure-activity relationships can be largely predicted based on lipophilicity calculations, although there are examples where inhibitors of both higher and lower potency than expected have been produced, which results from side-chain interactions with the enzyme [8]. Although the inhibitor precursors failed to significantly inhibit growth of prostate cancer cells, the study demonstrates the potential for development of rationally-designed drugs

Table 1

IC$_{50}$ values for inhibitor acyl-CoAs **1, 7a-o**, **10b**, as measured by the colorimetric assay [36], and the calculated lipophilicity (calc. LogP). IC$_{50}$ values are expressed as geometric means for three independent repeats with lower and upper geometric Standard Error of the Mean values in parentheses (see Experimental for details). [a]Only one IC$_{50}$ determination was performed for this compound due to the low synthetic yield of the acyl-CoA. [b]LogP values calculated using http://www.molinspiration.com/cgi-bin/properties.

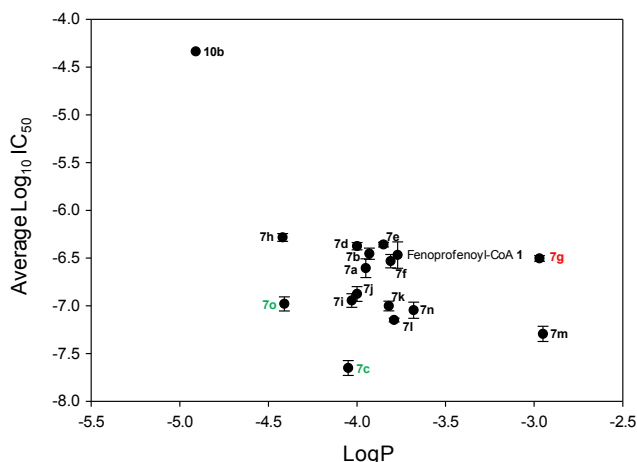| Fenoprofenoyl-CoA1	7a - n	10b		
Compound	R^1	R^2	IC$_{50}$ (nM)	Calc. logP[b]
1	–	–	340 (282, 408)	−3.77
7a	PhO	H	247 (217, 281)	−3.95
7b	H	PhO	354 (324, 379)	−3.93
7c	BnO	H	22.3 (20.1, 24.7)	−4.05
7d	3-MeOBnO	H	420 (399, 442)	−4.00
7e	Ph(CH$_2$)$_2$O	H	437 (423, 452)	−3.85
7f	3-MeOPh(CH$_2$)$_2$O	H	294 (267, 322)	−3.81
7g	3-F$_3$CPh(CH$_2$)$_2$O	H	313 (301, 325)	−2.97
7h	Me$_2$CHCH$_2$O	H	520 (492, 549)	−4.42
7i	H	BnO	113 (103, 124)	−4.03
7j	H	3-MeOBnO	133 (120, 147)	−4.00
7k	H	Ph(CH$_2$)$_2$O	99 (93, 106)	−3.82
7l	H	3-MeOPh(CH$_2$)$_2$O	71 (69, 73)	−3.79
7m	H	3-F$_3$CPh(CH$_2$)$_2$O	51 (46, 56)	−2.95
7n	H	Me(CH$_2$)$_4$O	90 (80, 100)	−3.68
7o	H	Me$_2$CHCH$_2$O	105 (95, 115)	−4.41
10b	H	PhO	4.6×10^4 [a]	−4.91

Fig. 1. Lipophilicity of 2-arylthiopropanoyl-CoA inhibitors **7a-o**, sulfone **10b** and fenoprofenoyl-CoA **1**. Log$_{10}$ IC$_{50}$ values are reported ± 1 SD. Calculated LogP used in this Figure are presented in Table 1. Compounds in green are more potent than expected, and compounds in red are less potent than expected based on calculated lipophilicity.

targeting AMACR.

4. Materials and methods

4.1. Sources of materials

Reagents were purchased from the Sigma-Aldrich Chemical Co., Fisher Scientific or Fluorochem. Reduced coenzyme A, tri-lithium salt was purchased from Calbiochem. Deuterated solvents were purchased from Goss Scientific. Anhydrous and general grade solvents were purchased from the Sigma-Aldrich Chemical Co. and used without further purification. Water for aqueous solutions was obtained from a Nanopure Diamond system (18.2 MΩ.cm^{-1}). Human recombinant AMACR 1A was expressed and purified and colorimetric substrate synthesized as previously described [36]. Fenoprofenoyl-CoA **1** was synthesized as previously described [10].

4.2. General experimental procedures

Syntheses were carried out at ambient temperature, unless otherwise specified. Solutions in organic solvents were dried with MgSO$_4$. Thin layer chromatography was performed on Merck silica aluminium plates 60 (F254) and visualized with UV light, potassium permanganate or phosphomolybdic acid. Column chromatography was performed using Fisher silica gel (particle size 35–70 μm). Purification of acyl-CoA esters was performed by solid-phase extraction using Oasis HLB 6 cc (200 mg) extraction cartridges. Columns were conditioned with acetonitrile and water. After loading the columns were eluted with water, 10%, 25% and 50% of MeCN in water (v/v). NMR spectra were recorded at 22 °C at 400.04 or 500.13 MHz (^{1}H) and 100.59 or 125.76 MHz (^{13}C) on Bruker Avance III NMR spectrometers in D$_2$O or CDCl$_3$. ^{1}H NMR spectra recorded for known compounds matched data reported in the literature unless otherwise stated. Mass spectra were recorded on an ESI-TOF instrument. High resolution mass spectra were recorded in ES mode. Acyl-CoA esters were characterized by ^{1}H NMR and HRMS. Compounds **1a** [50], **1b** [43], and **12b** [45] were synthesized by known literature methods. Compound **5b** was synthesized by the literature method [43] except that the temperature was increased to 280 °C.

Aqueous solutions for biological experiments were prepared in Nanopure water of 18.2 MΩ.cm^{-1} quality and were pH-adjusted with aq. HCl or NaOH. The pH of aqueous solutions was measured using a Corning 240 pH meter and Corning general purpose combination electrode. The pH meter was calibrated using Fisher Chemicals standard buffer solutions (pH 4.0 - phthalate, 7.0 - phosphate, and 10.0 - borate) at either pH 7.0 and 10.0 or 7.0 and 4.0. Calibration and measurements were carried out at ambient room temperature. Stock concentrations of acyl-CoA esters for assays were determined using ^{1}H NMR.

4.3. Synthesis of compounds

4.3.1. Ethyl (±)-2-(4-phenoxyphenylthio)propanoate (2b)

Anhydrous K_2CO_3 (367 mg, 2.65 mmol) was stirred with 4-phenoxybenzenethiol **1b** (534 mg, 2.64 mmol) in dry MeCN (4.9 mL) at $-45\,°C$ for 15 min. MeCN (1.8 mL) was added, followed by ethyl (±)-2-bromopropanoate (0.35 mL, 2.64 mmol) in MeCN (1.2 mL). The mixture was warmed to 20 °C and stirred for 20 h. Filtration (Celite®), evaporation and chromatography (PE/EtOAc 30:1) afforded **2b** (630 mg, 79%) as a colourless oil. IR νmax/cm^{-1} 1731 (C=O); ^{1}H NMR (400.04 MHz, CDCl$_3$): δ 7.44 (2H, d, $J = 8.8$ Hz), 7.37–7.31 (2H, m), 7.13 (1H, tt, $J = 7.3$, 1.1 Hz), 7.04–6.98 (2H, m), 6.93 (2H, d, $J = 8.8$ Hz), 4.18–4.07 (2H, m), 3.68 (1H, q, $J = 7.1$ Hz), 1.45 (3H, d, $J = 7.1$ Hz) and 1.20 (3H, t, $J = 7.1$ Hz); δ (100.59 MHz, CDCl$_3$) 172.47, 157.97, 156.30, 135.91, 129.76, 126.09, 123.75, 119.31, 118.61, 60.98, 45.69, 17.13 and 13.99; ESI–MS m/z 325.0991 [M + Na]+ (C$_{17}$H$_{18}$NaO$_3$S requires 325.0869). Compound **2b** was hydrolyzed to the corresponding acid using General Method C and converted into the acyl-CoA ester by General Method D. Spectroscopic data are available in the Supporting Information.

4.3.2. O-(3-Phenoxyphenyl) N,N-dimethylthiocarbamate (4a)

NaH (60% w/w dispersion in oil, 1.20 g, 30.0 mmol) was added slowly 3–phenoxyphenol **3a** (1.86 g, 10.0 mmol) in dry DMF (27 mL) at 10 °C. N,N-dimethylthiocarbamoyl chloride (5.78 g, 46.8 mmol) was added after evolution of H$_2$ had ceased. The mixture was stirred at 70 °C for 21 h and then cooled to ambient temperature. Water (100 mL) was added and the mixture was extracted thrice with CHCl$_3$. The organic layers were combined, washed with aq. KOH (0.89 M, 50 mL) and brine. Drying, evaporation and chromatography (PE/EtOAc 5:1) afforded **4a** (1.86 g, 68%) as a colourless oil. IR νmax/cm^{-1} 1139 (C=S); ^{1}H NMR (400.04 MHz, CDCl$_3$): δ 7.38–7.30 (3H, m), 7.15–7.05 (3H, m), 6.93 (1H, ddd, $J = 8.3$, 2.4, 1.0 Hz), 6.86 (1H, ddd, $J = 8.1$, 2.2, 1.0 Hz), 6.78 (1H, t, $J = 2.2$ Hz), 3.41 (3H, s), 3.26 (3H, s); δ (100.59 MHz, CDCl$_3$) 185.78, 157.41, 156.11, 154.50, 129.43, 129.31, 123.26, 118.76, 117.08, 115.53, 113.15, 42.76, 38.27; ESI-MS m/z 274.0900 [M + H]+ (C$_{15}$H$_{16}$NO$_2$S requires 274.0902).

4.3.3. S-3-Phenoxyphenyl-N,N-dimethylcarbamothioate (5a)

Compound **4a** (1.76 g, 6.43 mmol) was heated at 280 °C under Ar for 100 min. Chromatography (PE/EtOAc 19:1 → 3:1) gave **5a** (664 mg, 38%) as a brown oil. IR νmax/cm^{-1} 1671 (C=O); δ (400.04 MHz, CDCl$_3$) 7.39–7.30 (3H, m), 7.29–7.24 (1H, m), 7.20 (1H, dd, $J = 2.2$, 1.7 Hz), 7.13 (1H, tt, $J = 8.4$, 1.1 Hz), 7.09–7.01 (3H, m) and 3.03 (6H, br s); δ (100.59 MHz; CDCl$_3$) 166.01, 157.09, 156.46, 130.10, 129.98, 129.63, 129.59, 125.37, 123.32, 119.14, 118.91, 36.60; ESI-MS m/z 274.0879 [M + H]+ (C$_{15}$H$_{16}$NO$_2$S requires 274.0902).

4.3.4. Ethyl (±)-2-(3-phenoxyphenylthio)propanoate (2a).

Compound **5a** was prepared from 3–phenoxybenzenethiol **1a** (94 mg, 0.47 mmol), using the procedure as for compound **5b**, to afford a colourless oil (94 mg, 67%). IR νmax/cm^{-1} 1732 (C=O); ^{1}H NMR (400.04 MHz, CDCl$_3$): δ 7.38–7.30 (2H, m), 7.25 (1H, t, $J = 8.0$ Hz), 7.20–7.07 (3H, m), 7.04–6.97 (2H, m), 6.94–6.89 (1H, m), 4.15–4.03 (2H, m), 3.78 (1H, q, $J = 7.2$ Hz), 1.48 (3H, d, $J = 7.2$ Hz) and 1.17 (3H, t, $J = 7.2$ Hz); δ (100.59 MHz; CDCl$_3$) 172.38, 157.44, 156.59, 135.02, 129.88, 129.75, 126.95, 123.54, 122.24, 119.01, 118.04, 61.17, 44.94, 17.29 and 13.97; ESI-MS m/z 325.0868 [M + Na]+ (C$_{17}$H$_{18}$NaO$_3$S requires 325.0874).

4.3.5. Ethyl (±)-2-(3-phenoxyphenylsulfonyl)propanoate (8a)

A solution of OXONE® (470 mg, excess) in water (4.7 mL) was added to **2a** (115 mg, 0.38 mmol) in MeOH/THF (1:1, 4.7 mL) and the mixture was stirred for 4 h. The mixture was filtered (Celite®). Water was added to the evaporation residue, which was extracted thrice with EtOAc. Drying, evaporation and chromatography (PE/EtOAc 20:1 → 3:1) gave

8a (123 mg, 97%) as a colourless oil (> 95% pure by ^{1}H NMR), which was used in the next step without further purification. IR νmax/cm^{-1} 1738 (C=O), 1326, 1139 (S=O); δ (500.13 MHz, CDCl$_3$) 7.58 (1H, dt, $J = 7.5$, 1.1 Hz), 7.51 (1H, t, $J = 8.1$ Hz), 7.46 (1H, t, $J = 2.0$ Hz), 7.38 (2H, tt, $J = 7.5$, 2.0 Hz), 7.28 (1H, ddd, J = 8.2, 2.3, 1.0), 7.18 (1H, tt, $J = 7.4$, 0.9 Hz), 7.03 (2H, d, $J = 8.0$ Hz), 4.10 (2H, q, $J = 7.3$ Hz), 4.02 (1H, q, $J = 7.3$ Hz), 1.55 (3H, d, $J = 7.3$ Hz) and 1.17 (3H, t, $J = 7.3$ Hz); δ (125.76 MHz; CDCl$_3$) 165.96, 158.04, 155.65, 138.37, 130.34, 130.10, 124.52, 123.86, 123.42, 119.48, 118.53, 65.31, 62.21, 13.78 and 11.63; ESI-MS m/z 333.0799 [M - H]- (C$_{17}$H$_{17}$O$_5$S requires 333.0797).

4.3.6. Ethyl (±)-2-(4-phenoxyphenylsulfonyl)propanoate (8b)

Compound **2b** (143 mg. 0.47 mmol) was treated with OXONE®, as for **8a**, to give **8b** (129 mg, 82%) as a colourless oil. IR νmax/cm^{-1} 1738 (C=O), 1324, 1144 (S=O); ^{1}H NMR (400.04 MHz, CDCl$_3$): δ 7.81 (2H, d, $J = 9.0$ Hz), 7.45–7.37 (2H, m), 7.23 (1H, tt, $J = 7.4$, 1.1 Hz), 7.10–7.02 (4H, m), 4.14 (2H, q, $J = 7.2$ Hz), 4.02 (1H, q, $J = 7.1$ Hz, CH), 1.55 (3H, d, $J = 7.1$ Hz), 1.20 (3H, t, $J = 7.2$ Hz); δ (100.59 MHz, CDCl$_3$) 166.33, 162.94, 154.63, 131.66, 130.20, 130.02, 125.22, 120.45, 117.13, 65.44, 62.13, 13.82 and 11.90; ESI-MS m/z 333.0807 [M - H]- (C$_{17}$H$_{17}$O$_5$S requires 333.0797).

4.3.7. Ethyl (±)-2-(3-hydroxyphenylthio)propanoate (12a)

3-Hydroxythiophenol **11a** (3.00 g, 23.8 mmol) and ethyl 2-bromopropanoate (3.1 mL, 24 mmol) were stirred at reflux in CHCl$_3$ (80 mL) with NEt$_3$ (5.0 mL, 36 mmol) for 1 h. The cooled mixture was washed with water and brine. Drying, evaporation and chromatography gave **12a** (4.65 g, 86%) as a yellow oil. ^{1}H NMR (400.04 MHz, CDCl$_3$): δ 7.16 (1H, t, $J = 8.0$ Hz), 7.00 (1H, ddd, $J = 7.8$, 1.8, 1.0 Hz), 6.95 (1H, ddd, $J = 2.5$, 1.7, 0.4 Hz), 6.75 (1H, ddd, $J = 8.2$, 2.5, 1.0 Hz), 5.46 (1H, br s), 4.20–4.08 (2H, m), 3.81 (1H, q, $J = 7.1$ Hz), 1.50 (3H, d, $J = 7.1$ Hz), 1.20 (3H, t, $J = 7.1$ Hz). ^{13}C NMR (100.59 MHz, CDCl$_3$) δ 173.07, 155.89, 134.67, 129.90, 124.70, 119.23, 115.08, 61.42, 45.16, 17.36, 13.99. ESI-MS m/z 249.0577 [M + Na]+ (C$_{11}$H$_{14}$NaO$_3$S requires 249.0556).

4.3.8. General method A: Synthesis of compounds 2c, d, h, i, j, n and o

Compounds **12a** or **12b**, in DMF (15 mL), was stirred at 100 °C with K$_2$CO$_3$ (1.22 g, 8.84 mmol) and the appropriate alkyl bromide [benzyl bromide (0.32 mL; **2c** and **2i**), 3-methoxybenzyl bromide (0.32 mL or 0.37 mL; **2d** and **2j**), 1-bromo-2-methylpropane (0.29 mL; **2 h** and **2o**) or 1-bromopentane (0.24 mL; **2n**)] (2.66 mmol), for 1 h. The reaction mixture was cooled and the DMF was evaporated. The residue, in CH$_2$Cl$_2$, was washed twice with water and once with brine. Drying and evaporation gave the required compounds. Spectroscopic data for these compounds are given in the Supporting information.

4.3.9. General method B: Synthesis of compounds 2e-g, k-m

DIAD (0.65 mL, 3.31 mmol) was added dropwise to **12a** or **12b** (500 mg, 2.21 mmol), PPh$_3$ (869 mg, 3.31 mmol) and appropriate alcohol [2-phenylethanol (0.27 mL, 2.21 mmol, **2e**; or 0.17 mL, 1.46 mmol, **2k**), 2-(3-methoxyphenyl)ethanol (0.31 mL, 2.21 mmol, **2f**; 0.20 mL, 1.46 mmol, **2l**), or 2-(3-trifluoromethylphenyl)ethanol (0.33 mL, 2.21 mmol, **2g** and **2m**] in THF (10 mL), and the mixture was stirred for 18 h. Drying, evaporation and chromatography (PE/EtOAc 20:1) gave the esters **2e-g**, **k-m**. Spectroscopic data for these compounds are given in the Supporting information.

4.3.10. General method C: Hydrolysis of the ethyl ester to give compounds 6a-o

Aq. NaOH (2.5 M, 2.0 mL, 5.00 mmol) was stirred with of the ethyl ester **2a-o** in MeOH (15 mL) for 1.5 h. MeOH was evaporated and the mixture was acidified with aq. HCl (1.0 M) to pH ~ 3 and extracted twice with CH$_2$Cl$_2$. The combined organic layers were washed with brine. Drying, evaporation and chromatography (PE/EtOAc 2:1) gave

the carboxylic acids **6a-6o**. Spectroscopic data for these compounds are given in the Supporting information.

4.3.11. General method D: Formation of acyl-CoA esters **7a-o**

Carboxylic acids **6a-o** in dry CH_2Cl_2 (3.0 mL) was treated with N,N′-carbonyl diimidazole (32 mg, 0.2 mmol) in one portion and the mixture was stirred for 1 h. The mixture was washed with water (5 × 2 mL) and brine. Drying, filtration and evaporation gave the crude imidazolide. This material was dissolved in THF (3.0 mL), then CoA-Li$_3$ (31 mg, 0.04 mmol) in aq. NaHCO$_3$ (0.1 M, 3.0 mL) was added and the mixture was stirred for 18 h. The solution was acidified to pH ~ 3 with aq. HCl (1 M) and the solvents were partly removed under reduced pressure. Water (2.0 mL) was added and the mixture was washed with EtOAc (5 × 3 mL). Solid-phase extraction of the aqueous layer gave the required acyl-CoA esters **7a-o**. Spectroscopic data for these compounds are given in the Supporting Information.

4.3.12. Evaluation of AMACR inhibition by test compounds in vitro

Colorimetric assays were performed as previously described [30,36], except that the concentration of DMSO in the final assay was 10% (v/v). Control experiments showed that no reduction in activity was observed at this concentration. Assays were conducted in full- or half-volume 96-well plates, in a final volume of 200 or 100 μL, respectively. Control experiments have previously shown identical results using full and half-volume plates [30,36]. Reaction rates were determined by plotting A_{354} with time in Excel, and data was analysed using SigmaPlot 13 as previously described [30,36]. Log$_{10}$ IC$_{50}$ values were calculated from individual dose-response curves with inhibition of binding plotted against Log$_{10}$ drug concentration (in M). Mean Log$_{10}$ IC$_{50}$ values were then calculated from 3 independent repeats together with corresponding Log$_{10}$ Standard Error of the Mean (S.E.M.) values. Data were then converted to non-logarithmic values to produce the geometric mean with corresponding upper and lower limits of the geometric S.E.M. values (Table 1). K_i values were determined as described using 8 concentrations of substrate (150, 100, 66.6, 29.6, 19.8, 13.1, 8.8 and 5.9 μM in assay), in the presence of 0 or 22.5 nM **7c**, and analysed with SigmaPlot as previously described [36]. Data are means ± S.E.M., for 3 dependent repeats.

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Appendix A. Supplementary material

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