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A novel colorimetric assay for α -methylacyl-CoA racemase 1A (AMACR; P504S) utilizing the elimination of 2,4-dinitrophenolate†

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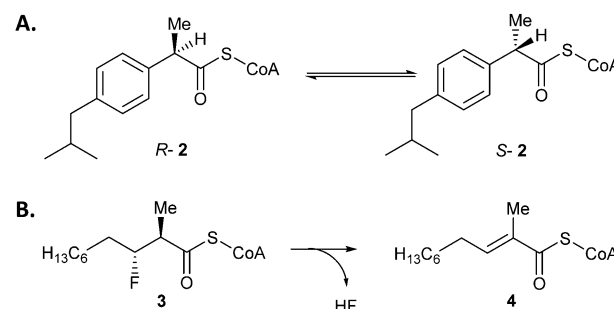
α -Methylacyl-CoA racemase (AMACR; P504S) regulates branched-chain fatty acid degradation, activates Ibuprofen and is a recognised cancer drug target. A novel, facile colorimetric assay was developed based on elimination of 2,4-dinitrophenolate. The assay was used to test 5 known inhibitors, determining IC₅₀ and K_i values, reversibility and characterizing irreversible inhibition.

α -Methylacyl-CoA racemase (AMACR;‡ P504S; E.C. 5.1.99.4) performs a key role in branched-chain fatty acid β -oxidation and the pharmacological activation of Ibuprofen and related drugs.^{1,2} AMACR catalyses its reaction by a deprotonation/reprotonation mechanism,^{3–5} in which either epimer of a 2-methylacyl-CoA or 2-arylpropanoyl-CoA substrate is converted to a near 1:1 mixture of epimers.^{3,5} Conversion of *R*-2-methylacyl-CoAs to their *S*-epimers enables degradation by β -oxidation.^{1,6} *R*-Ibuprofen and most related *R*-2-APA drugs¹ are pharmacologically activated by conversion to their corresponding acyl-CoA esters, before epimerization by AMACR. Hydrolysis of the epimeric products gives a mixture of *R*- and *S*-2-APA drugs, with the *R*-product recycled by the same pathway. Thus, inactive *R*-2-APA drugs are converted into their *S*-enantiomers, which are potent inhibitors of cyclooxygenase.⁷

Concentrations of AMACR are increased in all prostate cancers,^{8,9} and in several other cancers.^{10–13} Increased AMACR catalytic activity has also been reported in prostate cancer.^{14,15} Reducing the cellular AMACR 1A^{15–17} protein using siRNA or shRNA reduces proliferation of prostate cancer cell lines *via* a pathway which is synergistic with androgen withdrawal.¹⁵ Some advanced prostate cancer cell lines revert from androgen-independent to androgen-dependent status¹⁷ upon AMACR knock-down. Consequently, AMACR has attracted considerable interest as a novel prostate cancer drug target^{16,18–20} and biomarker.^{1,21}

Despite this interest, few inhibitors of AMACR have been reported. The majority of inhibitors are rationally designed acyl-CoA derivatives,^{18–20,22} which do not comply with Lipinski's guidelines²³ and need to be delivered as carboxylate prodrugs. Drug delivery can be limited by *in vivo* conversion of the prodrug to the acyl-CoA drug, although preliminary *in vivo* studies²⁴ appear promising. *N*-Dodecyl-*N*-methylcarbamoyl-CoA **1**, a transition-state analogue, is the most potent inhibitor reported to date.¹⁹ Several non-specific protein-modifying agents also inhibit AMACR.¹⁶ No structure–activity relationships have been reported, probably due to the difficulties in assaying enzyme activity.

AMACR catalyses the conversion of either *R*- or *S*-2-methylacyl-CoA esters into a near 1:1 mixture of epimers^{3,5} (Scheme 1A). Assaying AMACR activity is challenging due to the reversibility of the reaction and the difficulties in differentiating the epimeric products since the stereochemical centre undergoing a change in configuration is remote from the stereochemical centres in the CoA moiety. Several endpoint assays have been reported and used for inhibitor testing, such as those using chiral acyl-CoA substrates^{18,19} or their derivatives.²⁵ Notably, Wilson *et al.*¹⁶ reported screening



Scheme 1 Reactions catalysed by AMACR. (A) Conversion of *R*- or *S*-2-methylacyl-CoA into an epimeric mixture, as illustrated by ibuprofenoyl-CoA **2**. Assays commonly use an acyl-CoA substrate with α -³H which is 'washed out',¹⁶ or unlabelled substrate in the presence of ²H₂O followed by ¹H NMR analysis of the 'washed in' product.^{3,5} (B) The elimination of HF from (2*R*,3*R*)-3-fluoro-2-methyldecanoyl-CoA **3** to give unsaturated product **4**.

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† Electronic supplementary information (ESI) available: Experimental details including synthesis of compounds, kinetic plots, inhibitor testing. See DOI: 10.1039/c7cc00476a



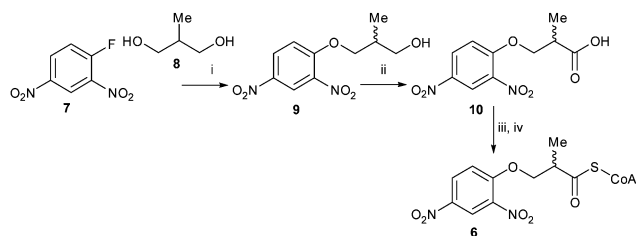
of a library of 5000 compounds using a modified radiolabelled assay, and identified several non-specific protein modifying agents that were potent inhibitors of AMACR. Their assay was used for kinetic analysis of AMACR, but it is an endpoint assay measuring substrate conversion at a single time-point and extensive manipulation of samples is required. In addition the assay is likely to be complicated by the presence of a kinetic isotope effect. A continuous circular dichroism (CD) assay for the closely related *Mycobacterium tuberculosis* enzyme (MCR), which follows the conversion of either *R*- or *S*-ibuprofenoyl-CoA 2 to a near racemic mixture,²⁶ has been used for limited testing of inhibitors.²² This assay is not subject to a kinetic isotope effect, but is low-throughput as only one sample can be analysed at the same time.

It has also been discovered that AMACR catalyses the elimination of fluoride from substrates such as 3-fluoro-2-methyldecanoyl-CoA (Scheme 1B), probably *via* an E1cb mechanism.²⁷ This reaction has the advantage of being irreversible, and the methyl peaks of substrate and product do not overlap in the ¹H spectrum allowing a simple route to determine the extent of conversion. This reaction has been used for the preliminary characterization of known inhibitors.²⁸

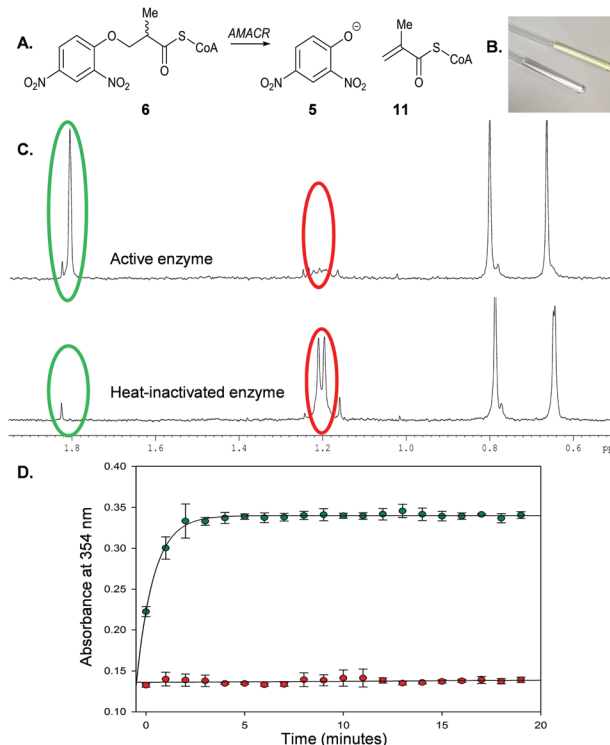
The elimination of fluoride from 3-fluoro-2-methyldecanoyl-CoA led us to consider whether a colorimetric leaving group might eliminate from a suitable substrate. The p*K*_a of HF (3.2)²⁹ and 2,4-dinitrophenol (3.93)³⁰ are similar, suggesting the conjugate bases will have similar leaving-group ability. Significantly, 2,4-dinitrophenolate 5 is a well-characterized chromophore ($\epsilon_{354} = 15\,300\text{ M}^{-1}\text{ cm}^{-1}$).³¹ AMACR is known to accept substrates with a wide variety of side-chain structures,¹ and hence the use of a substrate containing a 2,4-dinitrophenoxy- moiety as a chromogenic leaving group was investigated.

Racemic 3-(2,4-dinitrophenoxy)-2-methylpropanoyl-CoA 6 was synthesized (Scheme 2) by reaction of Sanger's reagent 7 with 2-methylpropan-1,3-diol 8 under basic conditions. Treatment of alcohol 9 with CrO₃ and H₂SO₄ resulted in the racemic acid 10. This was coupled with CoA-SH^{5,27,28} to give the desired substrate 6.

Incubation of 6 with active AMACR resulted in an elimination reaction (Scheme 3A). The sample containing active enzyme possessed an intense yellow colour, which was absent from negative controls (Scheme 3B). ¹H NMR analysis confirmed that 2,4-dinitrophenolate 5 and the predicted unsaturated product 11 were formed (Scheme 3C). Incubation of substrate 6 with active



Scheme 2 Synthesis of substrate 6. Reagents & conditions: (i) Na metal, 83%; (ii) CrO₃, conc. H₂SO₄, acetone, 67%; (iii) carbonyldiimidazole, CH₂Cl₂; (iv) CoA-SH (Li⁺)₃, 0.1 M NaHCO₃ aq./THF (1:1).



Scheme 3 Elimination of 2,4-dinitrophenolate 5 from 3-(2,4-dinitrophenoxy)-2-methylpropanoyl-CoA 6 by AMACR. (A) Reaction catalysed by enzyme; (B) NMR tubes showing samples containing active enzyme (yellow) and heat-inactivated enzyme (clear); (C) ¹H NMR spectra (500 MHz) of samples in B showing position of methyl groups of substrate 6 (red circles) and product 11 (green circles); (D) time course of reaction followed by monitoring absorbance of 2,4-dinitrophenolate 5 at 354 nm, showing negative control (heat-inactivated enzyme; red) and active enzyme (green). Assays comprised 40 μM 6 and recombinant AMACR (9.25 μg) in 100 μL NaH₂PO₄-NaOH, pH 7.4. Data are means ± SDM (*n* = 3).

enzyme in a microtitre plate led to a rapid increase in absorbance at 354 nm, which was not observed in controls containing heat-inactivated enzyme (Scheme 3D). This reaction occurred in an enzyme concentration-dependent manner (ESI,† Fig. S1 and S2).

Assay conditions were optimized (ESI,† Fig. S3–S5) by investigating the effect of various additives on enzymatic activity. Addition of 2-mercaptoethanol, dithiothreitol or BSA had no significant effect on activity. Addition of 0.1% (v/v) Triton X-100 to the assay mixture resulted in a modest reduction of activity. This is probably due to the formation of micelles, as the amounts used are above the reported critical micellar concentrations for Triton X-100 (0.19 and 1.25 mM).³² In contrast, addition of 1.5% (w/v) *N*-lauroyl-sarcosine reduced activity to levels observed in negative controls. The enzyme proved to be tolerant of DMSO (ESI,† Fig. S5), up to at least 8% (v/v) (~1.12 M). Michaelis-Menten kinetic behaviour was observed under the optimized assay conditions (ESI,† Fig. S6) and the following kinetic parameters were determined: $K_m = 58\text{ }\mu\text{M}$; $V_{max} = 112\text{ nmol min}^{-1}\text{ mg}^{-1}$; $k_{cat} = 0.088\text{ s}^{-1}$; $k_{cat}/K_m = 1517\text{ M}^{-1}\text{ s}^{-1}$. Optimised conditions for the assay were ~8 μg enzyme in 200 μL NaH₂PO₄-NaOH aq., pH 7.4 containing 40 μM 6 and up to 8% (v/v) DMSO at 30 °C, monitoring at 354 nm. Typical rates for active enzyme and negative controls



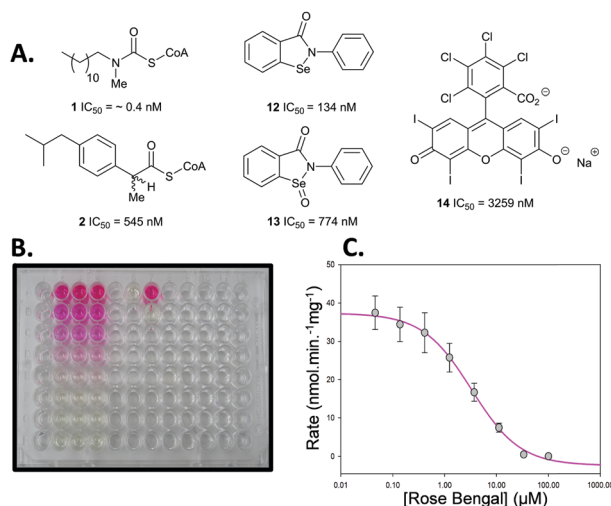


Fig. 1 Inhibitors used in assay. (A) Chemical structures and determined IC_{50} values from dose–response curves; (B) example assay in 96-well plate. The pink colour results from the presence of the inhibitor Rose Bengal **14** and the yellow colour from the 2,4-dinitrophenolate **5** produced by the enzyme from substrate **6**; (C) dose–response curve for Rose Bengal **14**.

(means \pm SD, $n = 3$) are 33 ± 1.9 and 0.72 ± 0.24 $nmol \cdot min^{-1} \cdot mg^{-1}$. The limit of detection is ~ 1.4 $nmol \cdot min^{-1} \cdot mg^{-1}$. The dynamic range is ~ 1.4 – 255 $nmol \cdot min^{-1} \cdot mg^{-1}$. The sensitivity threshold is ~ 0.5 – 1.0 $nmol \cdot min^{-1} \cdot mg^{-1}$. The Z' value³³ for the assay was 0.906 (see ESI,† for further details on how these values were calculated). The presence of other enzymes such as branched-chain acyl-CoA oxidases (ACOXs) could potentially generate a false positive signal, but this can be avoided by using *R-6* as ACOXs are known to be specific for *S-2*-methylacyl-CoA substrates.⁶

This new colorimetric assay for AMACR was validated using a series of known inhibitors for which bench-mark values are available (Fig. 1). *N*-Dodecyl-*N*-methylcarbamoyl-CoA **1** and ibuprofenoyl-CoA **2** were chosen as representative acyl-CoA inhibitors. Ebselen **12**, Ebselen Oxide **13** and Rose Bengal **14** were chosen as examples of the non-specific protein modifying agents reported by Wilson *et al.*¹⁶ Inhibitor potency was initially assessed using dose–response curves to determine IC_{50} values (see Fig. 1B and C for example).

N-Dodecyl-*N*-methylcarbamoyl-CoA **1** had a very low IC_{50} value of 0.4 nM determined by this assay, confirming its very high potency. This compares with the previous value of 98 nM, determined with an endpoint assay.¹⁹ Ibuprofenoyl-CoA **2** had a modest potency with an IC_{50} value of 554 nM. For both inhibitors activity was restored upon rapid dilution of the inhibitor, showing reversible inhibition. Michaelis–Menten kinetic analysis of compounds **1** and **2** showed that they were both competitive inhibitors of AMACR, with K_i values of 0.65 and 60 nM, respectively (Fig. 2 and ESI,† Fig. S7 and S8). It is noteworthy that both compounds appear to be much more potent when assayed using this method compared with other methods. K_i values of 98 nM and 56 μM were previously reported in the literature for *N*-dodecyl-*N*-methylcarbamoyl-CoA **1**¹⁹ and racemic ibuprofenoyl-CoA **2**³⁴ (with native rat enzyme), respectively. The reasons for the difference in apparent potency are not clear, but it probably

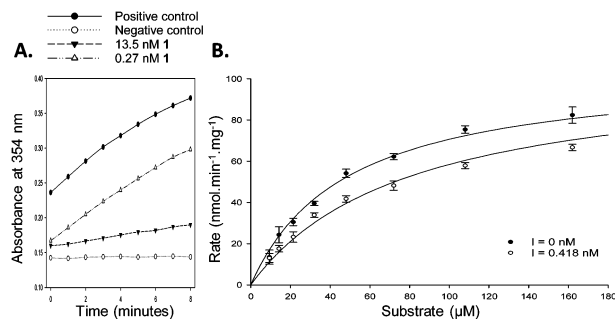


Fig. 2 Analysis of inhibitor **1**. (A) Reversibility experiment, showing enzymatic activity is restored upon dilution of concentrated enzyme with 13.5 nM **1** to 0.27 nM **1**; (B) kinetic analysis to determine K_i value for **1**.

results from differences in assay conditions including micelle formation by acyl-CoA substrates and inhibitors.

Analysis of the non-specific protein modification agents **12**, **13** and **14**, identified by Wilson *et al.*¹⁶ as AMACR inhibitors, confirmed their potency. Ebselen **12** gave an IC_{50} value of 133 nM, compared to the previously reported value¹⁶ of 2.789 μM . Rapid dilution of Ebselen **12** resulted in part restoration of activity in our assay (ESI,† Fig. S7), consistent with irreversible inhibition. This is consistent with the complex behaviour observed by Wilson *et al.*¹⁶

Similarly, Ebselen Oxide **13** was a potent inhibitor with an IC_{50} value of 774 nM compared to the previously reported value¹⁶ of 795 nM. Rapid dilution of **13** did not result in full restoration of activity, suggesting (covalent) irreversible inhibition. Further investigation showed that non-saturating time-dependent inhibition of AMACR occurred, with a second-order rate constant of 116 ± 8 $M^{-1} s^{-1}$ (Fig. 3 and ESI,† Fig. S10), consistent with **13** being a non-specific inhibitor operating by a one-step mechanism. Compound **14** was a reversible inhibitor ($IC_{50} = 3259$ nM), compared to 10 000 nM.¹⁶ Prolonged incubation caused photolytic protein degradation (ESI,† Fig. S9).

AMACR has attracted much attention as both a novel drug target and cancer biomarker since the first reports of its involvement in prostate cancer in 2003.¹⁵ Exploitation of this discovery has been very limited due to the absence of a suitable assay. Our novel colorimetric assay provides a versatile platform for detailed characterization of inhibitors and determination of

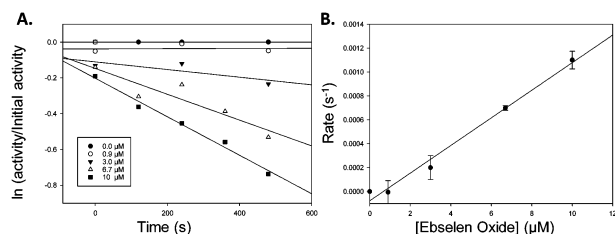


Fig. 3 Inactivation of AMACR by Ebselen Oxide **13**. (A) Time-dependent inactivation of recombinant AMACR by Ebselen Oxide **13**. Concentrations of Ebselen Oxide **13** (inset) are given for the pre-incubation phase before dilution of the enzyme with substrate **6** in the assay; (B) dependence of inhibition on Ebselen Oxide **13** concentration. First order rate constants (s^{-1}) derived from **A** was plotted vs. Ebselen Oxide **13** concentration in the pre-incubation phase. Data are mean \pm standard error.



structure–activity relationships. The assay also allows for inter-rogation of the complex biology of AMACR and its role in lipid metabolism and cancer. Similar colorimetric assays could potentially be adapted for use with other enzymes catalysing reactions *via* enolate intermediates, including other racemases,³⁵ acyl-CoA oxidases^{36,37} and other enzymes,^{38,39} several of which are of academic, medicinal, or biotechnological interest.

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Notes and references

‡ Abbreviations used: AMACR, α -methylacyl-CoA racemase splice variant 1A; 2-APA, 2-arylpropanoic acid (profen); BSA, bovine serum albumin; CoA, coenzyme A; MCR, 2-methylacyl-CoA racemase from *M. tuberculosis*.

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