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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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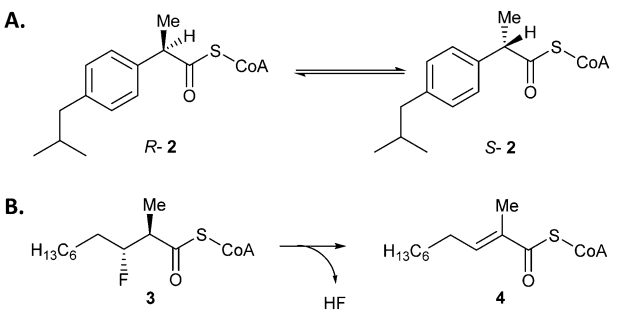
α-Methylacyl-CoA racemase (AMACR; P504S) regulates branched-chain fatty acid degradation, activates Ibuprofen and is a recognised cancer drug target. A novel, facile colorimetric assay was developed based on elimination of 2,4-dinitrophenolate. The assay was used to test 5 known inhibitors, determining IC_{50} 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 drugs[1] 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 status[17] 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 guidelines[23] 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* studies[24] 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-^{3}H which is 'washed out',[16] or unlabelled substrate in the presence of ^2H_2O followed by ^{1}H 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

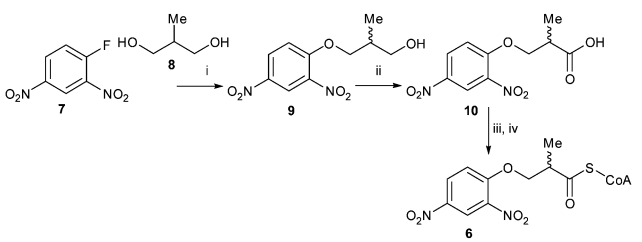
of a library of 5000 compounds using a modified radiolabelled assay, and identified several non-specific protein modifying agents that were potent inhibitors of AMACR. Their assay was used for kinetic analysis of AMACR, but it is an endpoint assay measuring substrate conversion at a single time-point 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 ^{1}H spectrum allowing a simple route to determine the extent of conversion. This reaction has been used for the preliminary characterization of known inhibitors.[28]

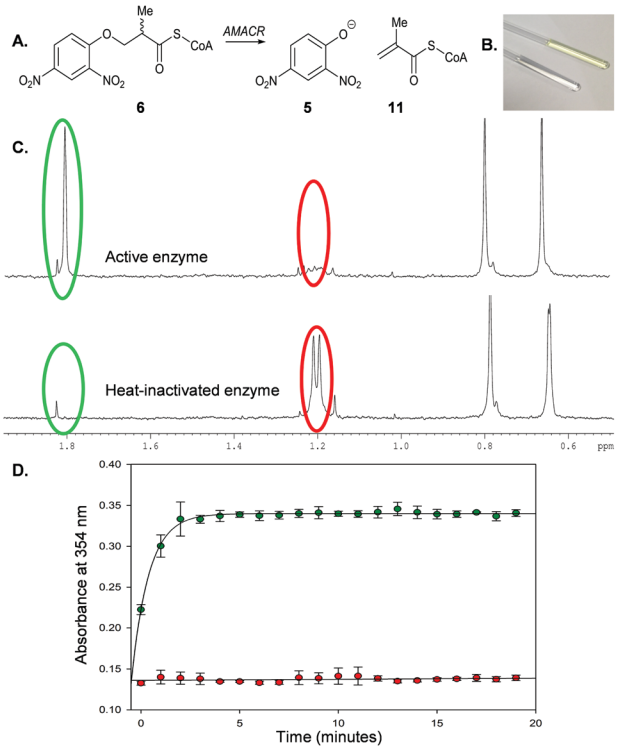
The elimination of fluoride from 3-fluoro-2-methyldecanoyl-CoA led us to consider whether a colorimetric leaving group might eliminate from a suitable substrate. The pK_a of HF (3.2)[29] and 2,4-dinitrophenol (3.93)[30] are similar, suggesting the conjugate bases will have similar leaving-group ability. Significantly, 2,4-dinitrophenolate **5** is a well-characterized chromophore (ε_{354} = 15 300 M^{-1} 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 CrO_3 and H_2SO_4 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). ^{1}H 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) CrO_3, conc. H_2SO_4, acetone, 67%; (iii) carbonyldiimidazole, CH_2Cl_2; (iv) CoA–SH (Li^+)$_3$, 0.1 M $NaHCO_{3\,aq.}$/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) ^{1}H 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-nitrophenoxide **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 NaH_2PO_4–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 μM; V_{max} = 112 nmol min^{-1} mg^{-1}; k_{cat} = 0.088 s^{-1}; k_{cat}/K_m = 1517 M^{-1} s^{-1}. Optimised conditions for the assay were ~8 μg enzyme in 200 μL NaH_2PO_4–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

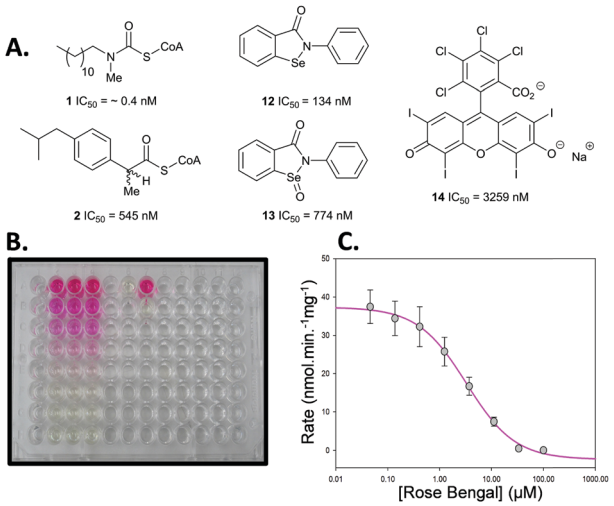


Fig. 1 Inhibitors used in assay. (A) Chemical structures and determined IC_{50} values from dose–response curves; (B) example assay in 96-well plate. The pink colour results from the presence of the inhibitor Rose Bengal **14** and the yellow colour from the 2,4-dinitrophenolate **5** produced by the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

(means ± SD, $n = 3$) are 33 ± 1.9 and 0.72 ± 0.24 nmol min^{-1} mg^{-1}. The limit of detection is ~1.4 nmol min^{-1} mg^{-1}. The dynamic range is ~1.4–255 nmol min^{-1} mg^{-1}. The sensitivity threshold is ~0.5–1.0 nmol min^{-1} mg^{-1}. The Z'* value[33] 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

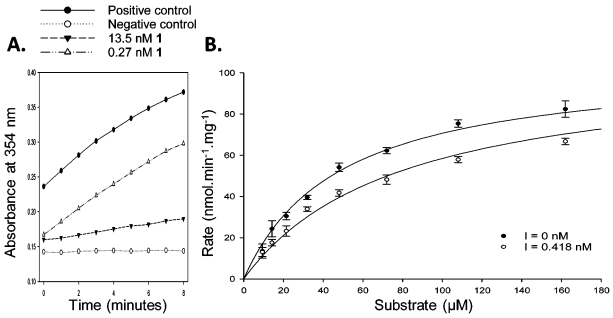


Fig. 2 Analysis of inhibitor **1**. (A) Reversibility experiment, showing enzymatic activity is restored upon dilution of concentrated enzyme with 13.5 nM **1** to 0.27 nM **1**; (B) kinetic analysis to determine K_i value for **1**.

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 value[16] 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 value[16] 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

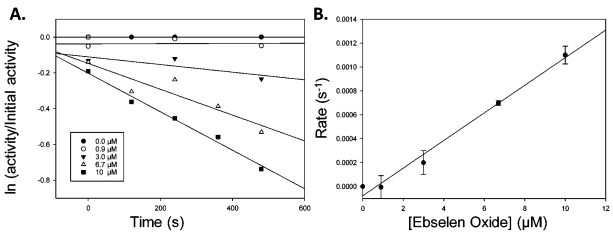


Fig. 3 Inactivation of AMACR by Ebselen Oxide **13**. (A) Time-dependent inactivation of recombinant AMACR by Ebselen Oxide **13**. Concentrations of Ebselen Oxide **13** (inset) are given for the pre-incubation phase before dilution of the enzyme with substrate **6** in the assay; (B) dependence of 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 ± standard error.

structure–activity relationships. The assay also allows for interrogation 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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