Introduction
Branch-chain fatty acids are common in the diet and similar structures are found in medicines such as ibuprofen and related drugs. Metabolism of branch-chain fatty acids requires that the centres bearing the methyl groups possess S-stereochemical configuration, but those with R-configuration are produced in the body and are found in the diet. Ibuprofen and related drugs require S-configuration for their anti-inflammatory properties, but these drugs are usually given as a mixture of R- and S-enantiomers. The enzyme α-methylacyl-CoA racemase (AMACR) catalyses R- to S- conversion of 2-methylacyl-CoA derivatives of fatty acids enabling β-oxidation. Similarly, acyl-CoA derivatives of ibuprofen and similar drugs are converted, resulting in pharmacological activation. 1, 2

AMACR levels are increased in all prostate cancers, some colon cancers and other cancers. 1, 3 In prostate cancer, higher AMACR levels result in higher proliferation rates 4 and androgen-independent growth 5 and AMACR is recognised as a novel drug target. However, few inhibitors have been identified, largely due to the difficulties in measuring enzyme activity which makes it difficult to quantify drug potency. 1 AMACR catalyses the irreversible elimination of hydrogen fluoride from 3-fluoro-2-methylacyl-CoA substrates, 6 but translating this reaction to a convenient colorimetric or fluorometric assay has proven difficult. 8 4-Nitrophenol derivatives are commonly used as colorimetric substrates for enzymes. This study reports the synthesis of a 2,4-dinitrophenol-containing AMACR substrate and the characterisation of known AMACR inhibitors using a convenient colorimetric microtitre plate assay.

Results and Discussion
2,4-Dinitrophenol is fully ionised at neutral pH giving a yellow colour and has a similar pKα to HF, which is eliminated from known AMACR substrates. Therefore an acyl-CoA derivative containing 2,4-dinitrophenol was investigated. Reaction of 2 with alcohol 3 to give 4 followed by oxidation gave the racemic acid 5, which was converted to the desired substrate 1 (Scheme 1). Incubation of 1 with recombinant human AMACR 1A resulted in formation of unsaturated product 6 and 2,4-dinitrophenol 7 resulting in a yellow colour.

Scheme 1: Synthesis of novel substrate 1 and reaction with AMACR.

AMACR was active around neutral pH and retained full activity in the presence of 8% (v/v) DMSO. Kinetic analysis of substrate 1 showed that Michaelis-Menten kinetics were observed (Figure 1), with the following parameters: 

Km = 56 ± 4.5 μM; Vmax = 112 ± 4 nmol.min⁻¹.mg⁻¹; kcat = 0.868 s⁻¹; kcat/Km = 1571 s⁻¹.M⁻¹. This shows that substrate 1 is converted with 446% of the efficiency of 3-fluoro-2-methylacyl-CoA and was significantly more efficient than ‘racemisation’ of 2-methyldecanoyl-CoA (as judged by kcat/Km). 6

Figure 1: Kinetic analysis for substrate 1.

The known inhibitor Rose Bengal 2 was tested to validate the method for characterisation of inhibitors. 2 A dose-response curve was efficiently produced using a microtitre plate assay.

Figure 2: AMACR inhibition assay using Rose Bengal as an inhibitor. A. 96-Well plate showing colour change; B. Dose-response curve for Rose Bengal.

A number of other known AMACR inhibitors and substrates were tested using a dose-response curve at a fixed substrate concentration of 40 μM. Ibuprofenoyl-CoA and related compounds are known substrates and should behave as competitive inhibitors. All of these compounds inhibited the enzyme with IC50 values of ca. 300-500 nM. 2-Methyldecanoyl-CoA also inhibited the reaction, and was ca. 4x more potent than decanoyl-CoA. Inhibition was decreased in acyl-CoA esters with shorter alky chains. The best acyl-CoA inhibitor was N-dodecyl-N-methylcarbamoyl-CoA, which was ~500 – 1000 x more potent than the other acyl-CoA inhibitors (as judged by IC50 values). The non-specific protein modifying reagents reported by Wilson et al. also inhibited the enzyme; in contrast to previous reports Ebselen behaved as a time- and concentration-dependent inactivator with a rate constant of 114 M⁻¹ s⁻¹.

Figure 3: Selected acyl-CoAs and protein modifying agents shown to inhibit the conversion of substrate 1 to 6 and 7 by AMACR using the colorimetric assay.

Conclusions
The colorimetric substrate 1 provides a convenient method for assaying AMACR and determining the behaviour and potency of inhibitors. AMACR is a promising drug target for prostate and other cancers; but until now it has been under-exploited because of the difficulties in determining enzyme activities. Inhibitors previously reported in the literature are largely rationally designed acyl-CoA esters, which do not comply with Lipinski guidelines. 8 This new assay will facilitate the testing and development of drugs by structure-based design, rational design and lends itself to screening approaches. The latter should allow identification of inhibitors with good drug-like properties.

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References