Introduction

 Branched-chain fatty acids are common in the diet and similar structures are found in medicines such as Ibuprofen and related drugs. Metabolism of branched-chain fatty acids requires that the centres bearing the methyl groups possess S-stereochirnical 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 (Scheme 1) enabling β-oxidation. Similarly, acyl-CoA derivatives of Ibuprofen and similar drugs are converted, resulting in pharmaceutical 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 and androgen-independent growth5 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 (Scheme 1),2 but translating this reaction to a convenient colorimetric or fluorometric assay has proven difficult.3 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 pKa to HF, which is eliminated from known AMACR substrates. Therefore an acyl-CoA derivative containing 2,4-dinitrophenol was sought. Reaction of 2-wafer-thick 1.85 g of 3-fluorooctanoyl-2,4-dinitrophenol with potassium hydroxide at 45 °C (Scheme 2) to give 4 followed by oxidation gave the racemic acid 5, which was converted to the desired substrate 1 (Scheme 2). 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 2: Synthesis of novel substrate 1 and reaction with AMACR. Reagents & conditions: i. Na metal; ii. Jones oxidation; iii. COD, DCM; iv. CoA-SH, NaHCO3, aq/THF (1:1); v. NaH2PO4-NaOH, pH 7.2, ca. 70% H2O. 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, with the following parameters: \( K_m = 56.4 \pm 4.5 \) M; \( V_{max} = 112 \pm 4 \) nmol.min\(^{-1}\).mg\(^{-1}\); \( k_{cat}/K_m = 1517 \pm 1 \) s\(^{-1}\). This shows that substrate 1 is converted with ~44% of the efficiency of 3-fluoro-2-methyldecanoyl-CoA and was significantly more efficient than ‘racemisation’ of 2-methyldecanoyl-CoA (as judged by \( k_{cat}/K_m \)).

The known inhibitor Rose Bengal2 was tested to validate the method for characterisation of inhibitors (Figure 1). A dose-response curve was efficiently produced using a microtitre plate assay.

A Convenient Colorimetric Assay for α-Methylacyl-CoA Racemase (AMACR; P504S) and Testing Of Inhibitors

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Figure 1: 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. 400-800 nM. 2-Methylenedecanoyl-CoA also inhibited the reaction, and was ca. 3x more potent than decanoyl-CoA. Inhibition was decreased in acyl-CoA esters with shorter alkyl chains. The best acyl-CoA inhibitor was N-dodecyl-N-methylcarbamoyl-CoA,6 which was ~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.10 also inhibited the enzyme; in contrast to previous reports Ebselen oxide behaved as a time- and concentration-dependent inactivator with a rate constant of 116 M\(^{-1}\) s\(^{-1}\).

Scheme 1: AMACR catalysed ‘racemisation’ and elimination reactions.

- Normal AMACR activity
- Unusual AMACR activity

References

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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 limited to rationally designed acyl-CoA esters, which do not comply with Lipinski guidelines. 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.