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Introduction

Branch-chain fatty acids are common in the diet and similar structures are found in medicines such as Ibufrofen 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. Ibufrofen 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 the S- to R-conversion of 2-methylacyl-CoA derivatives of fatty acids enabling β-oxidation. Similarly, acyl-CoA derivatives of Ibufrofen 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 rates1 and androgen-independent growth2 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.5 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 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. Reagents & conditions: i. Na metal; ii. Jones oxidation; iii. CDI, DCM; iv. CoA-SH, NaHCO3 aq./THF (1:1); v. NaH2PO4-NaOH, pH 7.4, ca. 77% 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 (Figure 1), with the following parameters: $K_m = 56 \pm 4.5 \mu M$; $V_{max} = 112 \pm 4 \text{ nmol.min}^{-1} \text{mg}^{-1}$; $k_{cat} = 0.088 \text{ s}^{-1}$; $k_{cat}/K_m = 1571 \text{ s}^{-1} \text{M}^{-1}$. This shows that substrate 1 is converted with >450% of the efficiency of 3-fluoro-2-methylacyl-CoA and was significantly more efficient than ‘racemisation’ of 2-methyldecanoyl-CoA (as judged by $k_{cat}/K_m$).6

Figure 1: Kinetic analysis for substrate 1.

The known inhibitor Rose Bengal7 was tested to validate the method for characterisation of inhibitors. (Figure 2). A dose-response curve was efficiently produced using a microtitre plate assay.

AMACR inhibition is achieved by two different drug classes. One class inhibits catalysis by binding to the active site and the other class inhibits catalysis by binding to the allosteric site. The two classes of inhibitors act on the enzyme in a mutually exclusive manner. The binding of the allosteric inhibitors to the enzyme does not prevent the binding of the active site inhibitors. The active site inhibitors are effective in blocking the enzyme at micromolar concentrations, whereas the allosteric inhibitors are effective only at submicromolar concentrations. The allosteric inhibitors are known to inhibit the enzyme at concentrations of 10 µM or less, whereas the active site inhibitors are effective at concentrations of 100 µM or less.

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 to 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