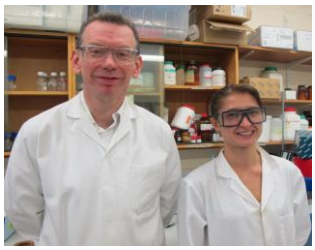


High-throughput screening to identify novel inhibitors of human α-methylacyl-CoA racemase 1A (AMACR; P504S)

Student: Yoana Petrova. Supervisor: Dr. Matthew Lloyd.

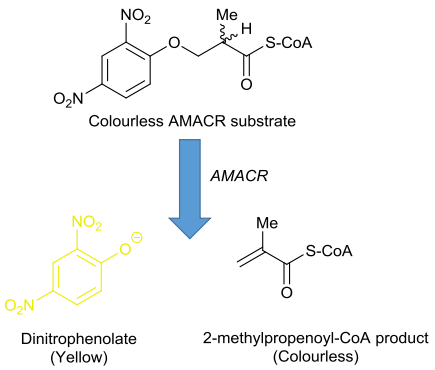
Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, U.K.

Introduction

Pristanic acid [2*R*,*S*,6*R*,10*R*-(2,6,10,14-tetramethyl)pentadecanoic acid] is a 2-methyl saturated fatty acid derived from phytanic acid [3*R*,*S*,7*R*,11*R*-(3,7,11,15-tetramethyl)hexadecanoic acid] by α-oxidation in the body. Phytanic acid is obtained from the diet and is particularly abundant in foods such as red meat and dairy products.[1] The α-oxidation of phytanic acid produces a mixture of 2*R*- and 2*S*-methyl pristanic acid epimers.[2] However, the acyl-CoA oxidases responsible for the further metabolism of pristanic acid by β-oxidation have the absolute requirement for the 2*S*-methyl acyl-CoA esters.[3] The enzyme involved in the conversion of the 2*R* to the 2*S* isomer is α-methylacyl-CoA racemase (AMACR).[4] AMACR has also the enzyme responsible for the activation of Ibuprofen in the pathway, converting the *R*-Ibuprofen to the pharmacologically active *S*-Ibuprofen.[5] The reactions catalysed by AMACR proceed via an enolate intermediate.[6]

AMACR levels are increased (up to 9 fold) in all types of prostate cancers and phytanic acid has been shown to have a role in regulating levels.[7,8] Studies have shown a correlation between the consumption of products high in phytanic acid (red meat and dairy products) and advanced prostate cancer.[9,10] It was demonstrated that reducing AMACR levels using siRNA reduced the proliferation of prostate cancer cells, making AMACR an attractive target.[7]

There are two main problems hindering the discovery of inhibitors against AMACR: the lack of a convenient assay to measure the activity of the enzyme and the fact that the CoA moiety is essential for binding of the inhibitor. Many CoA analogues were synthesised and showed inhibition of AMACR, however these are not drug-like molecules due being zwitterionic and having high MWs. The first problem was resolved when a colorimetric assay was developed by Dr. Maksims Yevglevskis (unpublished work), who is part of Dr. Lloyd's research group. The colorimetric assay uses the reaction, in which a colourless acyl-CoA substrate is converted to a yellow product by AMACR (Scheme 1). The formation of the yellow product allows the activity of the enzyme to be assayed in 96-well plates using a spectrophotometer. The second problem concerning the lack of drug-like inhibitors for AMACR is addressed in the aims of this study.



Scheme 1 *The scheme shows the novel E1cB reaction catalysed by AMACR. The colourless acyl-CoA substrate is converted to a yellow 2,4-dinitrophenolate product and a colourless acyl-CoA product in one-step irreversible reaction.*

Aims and objectives

The aim of the project is to discover a novel class of small molecule compounds, which inhibit the activity of AMACR and exhibit drug-like properties. The objectives were to: 1) Optimise the colorimetric assay conditions for high throughput screening of compounds against AMACR; 2) Complete high throughput screening of 7680 compounds and identify potential inhibitors ('hits'); 3) Further characterise the 'hits' with respect to their inhibitor properties.

Materials and Methods

All materials and reagents are obtained from the Sigma-Aldrich Chemical Co. or Fisher Scientific Ltd unless otherwise stated. The compound libraries were obtained from MRC Technology.

AMACR expression, extraction and purification

Competent *E. coli* Rosetta2 (DE3) cells (Novagen) were prepared and transformed with plasmid encoding for human His-tag AMACR enzyme[6] using the $CaCl_2$-heat shock method. Recombinant cells expressing the enzyme were lysed with the One Shot cell disruption system. The AMACR enzyme was purified by metal chelate chromatography. SDS-PAGE was performed to confirm the presence of the enzyme in fractions. Visking tubing dialysis was used to buffer exchange the enzyme, and the concentration of the enzyme was determined by measuring the absorbance at 280 nm using a Helios Omega spectrophotometer .[11]

High throughput screening assay conditions

The assay was performed in 96-well half-area plates. The activity of the enzyme was assayed by measuring the absorbance at two wavelengths (354nm and 390nm) using a BMG LabTech FluoStar Omega spectrophotometer. Measurements were taken every minute for 8 minutes. The total assay volume used was 100µL. The library compounds were used at 30µM in the assay, giving a final DMSO concentration in the assay of 3% (v/v). The library compounds were incubated with the AMACR enzyme for 10 minutes before addition of the substrate. The substrate concentration in the assay was 18µM, which is equal to the K_m value (unpublished work). The enzyme was used at 0.086mg.mL^{-1} in the assay, which is within the concentration range previously used in Dr. Lloyd's group. The positive control had 3% (v/v) DMSO to replace the library compound and the negative control had phosphate buffer to replace the enzyme and 3% (v/v) DMSO to replace the inhibitor.

IC_{50} determination

The IC_{50} determination was performed on inhibitors identified from the screening under the same assay conditions, with a substrate concentration of 40µM in the final assay. The top concentration of the selected inhibitors in the assay was 30µM. A 3-fold dilution series was used with 8 concentrations of inhibitor in total. All dilutions of the inhibitors were performed in DMSO in order to avoid precipitation of the inhibitors.

Reversibility experiments

The assay conditions were consistent with those used in the IC_{50} determination. The inhibitors were incubated with the concentrated enzyme for 10 min. The inhibitor concentration in the inhibitor-enzyme mixture was 30µM. Before the addition of the substrate, the inhibitor-enzyme mixture was diluted with phosphate buffer to give 0.086mg.mL^{-1} enzyme and 0.66µM of inhibitor in the final assay. Rates were determined as above

Results and Discussion

High throughput screening

Possible inhibitors ('hits') were identified by comparison of the absorbance trace to positive and negative controls, and were taken forwards for IC_{50} determination. Approximately 70 'hits' were identified during the screening, giving a 'hit' rate of 0.9%, which is consistent with the hit rate of <1% expected from a diverse and unbiased library. [12]

IC_{50} determination

The results from the IC_{50} determination are displayed in Figure 1.

The top concentration of inhibitors used in the IC_{50} determination was 30µM due to the availability of limited amounts of compound. This is approximately 3 times lower than the top concentration of 100µM used previously in the IC_{50} determination of various CoA analogues, known competitive inhibitors of the AMACR. The IC_{50} values reported for some rationally designed 2-methylacyl-CoA analogues, including *R*- and *S*-Ibuprofenoyl-CoA, ranged between 0.5µM and 20µM.[13, 14] The inhibitors reported in this study have IC_{50} values in that range, suggesting that they have similar potency to the known inhibitors of the enzyme.

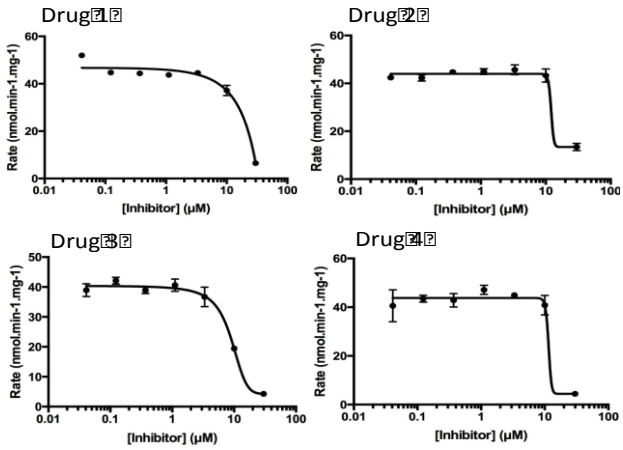


Figure 1 *IC_{50} curves for four selected inhibitors (drugs 1-4). The IC_{50} values are : 16.2*µM (Drug 1), 15.2µM (Drug 2) 9.3µM (Drug 3) and 12.8µM (Drug 4).

Reversibility experiments

The reversibility experiments were performed in order to define the mechanism of inhibition (Figure 2). The experiment required incubation of the enzyme with inhibitor at a concentration 10 times the IC_{50}.[12] However, due to insufficient amount of compound, 30µM of inhibitor was used, approximately 2-3x IC_{50}. It can be seen that the progress curve for the diluted inhibitors (0.66µM) is parallel to the one for the positive control, suggesting a reversible mode of inhibition because the activity of the enzyme is fully restored upon diluting the inhibitor to 0.04-0.07x IC_{50}. The reversibility experiments also suggest that the compounds identified are enzyme inhibitors as opposing to non-specific denaturing agents.

Future Directions

The future directions of the project will involve performance of the IC_{50} determination and reversibility experiments to all of the 'hits' identified from the screening. Furthermore, comparisons

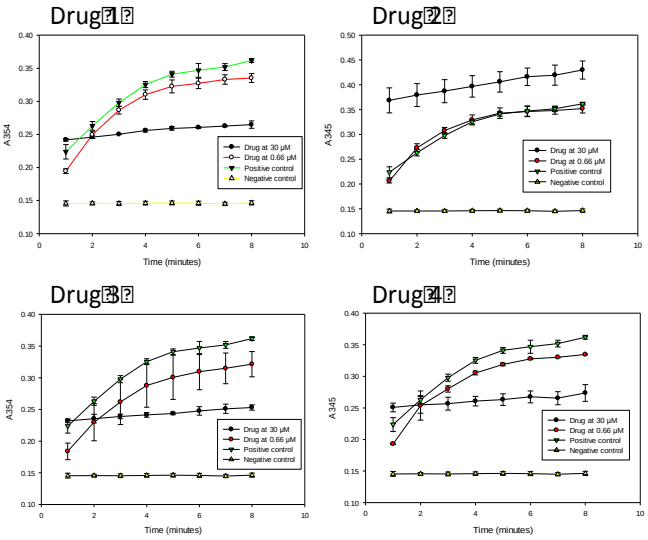


Figure 2 *Reversibility experiments curves for four selected inhibitors (drugs 1-4). The positive control is shown in red and the negative control in yellow. The black line represents the drugs at* 30µM *and the green line the diluted drug at 0.66*µM.

between the chemical structures of the inhibitors by computational chemistry methods will allow the identification of a common pharmacophore and the synthesis of analogues with improved activity. The development of a fluorescent assay is another future goal, as it will allow the measurement of the activity of AMACR in cell cultures and the evaluation of the inhibitors *in vivo.*

Deviations from original project

The original proposal for the project aimed for developing a convenient assay for measuring the activity of the AMACR. The reaction shown in Scheme 1 had been characterised by Dr. Lloyd's research group at the time the project proposal was drafted. Therefore, the determination of the kinetic parameters of known inhibitors and the use of the assay to determine the inhibitors' potency were part of the original project proposal. However, by the time I started the project, that part of the work in the proposal had been completed and the project I embarked on was a continuation of the original one.

Value of the studentship to the student

The 8-week placement funded by the Biochemical Society allowed me to gain a valuable experience in variety of laboratory techniques such as: preparation of competent cells; protein expression using recombinant DNA technology; protein purification using His-tag; SDS-PAGE analysis. I was actively involved in the optimisation of the assay conditions for high throughput screening and in the process of screening. The placement gave me the opportunity to talk to other members of the team and to learn what the life of a researcher entails. As a consequence of the 8 weeks in the lab, I will definitely be considering a PhD after graduation.

References

1) Lloyd*, et al.*, *Prog. Lipid Res.*, **2013**, 52, 220-230;
2) Ackman, *et al., Lipids,* **1967**, 2, 357-362;
3) Battaile, *et al., Lipid Metab.,* 1998, 1390, 333-338;
4) Schmitz, *et al., Eur. J. Biohem.,* **1995**, 231, 815-822;
5) Woodman, *et al., Chem. Commun.,* **2011**, 47, 7332-7334;
6) Darley, *et al., Org. Biomol. Chem.,* **2009**, 7, 543-552;
7) Zha*, et al.*, *Cancer Res.*, **2002**, 62, 2220-2226;
8) Mobley, *et al., Cancer Epidemiol. Biomarkers Prev.,* **2003**, 12, 775-783;
9) Wright, *et al., Prostate,* **2011**, 71, 498-506;

10) Wright, *et al., Int. J. Cancer,* **2012**, 131, 1396-406;
11) Yevglevskis*, et al.*, *Chem. Commun.*, **2014**, 50, 14164-14166;
12) Copeland, *Evaluation of enzyme inhibitors in drug discovery A guide for medicinal chemists and pharmacologists,* John Wiley&Sons, **2005**;
13) Carnell, *et al., J. Med. Chem.,* **2007**, 50, 2700-2707;
14) Carnell, *et al., ChemMedChem.,* **2013**, 8, 1643-1647.