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Structure-activity relationships of rationally designed AMACR 1A inhibitors

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ABSTRACT

α-Methylacyl-CoA racemase (AMACR; P504S) is a promising novel drug target for prostate and other cancers. Assaying enzyme activity is difficult due to the reversibility of the 'racemisation' reaction and the difficulties in the separation of epimeric products; consequently few inhibitors have been described and no structure–activity relationship study has been performed. This paper describes the first structure–activity relationship study, in which a series of 23 known and potential rational AMACR inhibitors were evaluated. AMACR was potently inhibited (IC_{50} = 400–750 nM) by ibuprofenoyl-CoA and derivatives. Potency was positively correlated with inhibitor lipophilicity. AMACR was also inhibited by straight-chain and branched-chain acyl-CoA esters, with potency positively correlating with inhibitor lipophilicity. 2-Methyldecanoyl-CoAs were *ca.* 3-fold more potent inhibitors than decanoyl-CoA, demonstrating the importance of the 2-methyl group for effective inhibition. Elimination substrates and compounds with modified acyl-CoA cores were also investigated, and shown to be potent inhibitors. These results are the first to demonstrate structure–activity relationships of rational AMACR inhibitors and that potency can be predicted by acyl-CoA lipophilicity. The study also demonstrates the utility of the colorimetric assay for thorough inhibitor characterisation.

1. Introduction

Branched-chain fatty acids (*e.g.* phytanic acid pristanic acids) are common components of the human diet, and derivatives of such compounds are used as drug molecules *e.g.* ibuprofen [1,2]. Degradation of branched-chain fatty acids occurs as the acyl-CoA ester, and the acyl-CoA oxidases and other enzymes involved in β-oxidation have an absolute requirement for *S*-2-methylacyl-CoAs [3–5]. However, *R*-2-methylacyl-CoAs are produced from dietary and endogenous fatty acids and these cannot be immediately degraded by β-oxidation. The enzyme α-methylacyl-CoA racemase [1,2] (AMACR; P504S; E.C. 5.1.99.4) catalyses conversion of *R*-2-methylacyl-CoAs to a near 1:1 epimeric mixture [6,7] by a deprotonation/reprotonation reaction [7,8], probably *via* an enolate intermediate [9] (this reaction is referred to as "racemisation" [10]). The resulting *S*-2-methylacyl-CoAs are degraded by β-oxidation whilst the 2*R* epimers are further processed to the 2*S* epimers by AMACR [1,2]. AMACR also plays a key role in the *in vivo* pharmacological activation of *R*-ibuprofen by conversion to *S*-ibuprofen, via the corresponding acyl-CoA esters [1,2,11]. The *S*-ibuprofen resulting from this pathway exerts its anti-inflammatory effect by inhibiting cyclooxygenase-1 and -2 [12].

AMACR protein levels are increased in prostate [13,14] and several other cancers [1,15–18]. Catalytic activity of AMACR is increased by 4- to 10-fold in prostate cancer cells [19,20], with the AMACR 1A splice variant [1,2,21–24] (possessing "racemase" activity [7,10]) showing the most significant increase in expression [19,20]. Reducing AMACR 1A levels using siRNA or shRNA approaches [19,25,26] has been shown to reduce proliferation of prostate cancer cells *via* a pathway which is synergistic with the use of an androgen receptor antagonist, studies which have validated AMACR 1A as a chemotherapeutic target. Some advanced prostate cancer cell lines revert from castrate-resistant (a.k.a. androgen-independent) growth to androgen-dependent growth upon knockdown of AMACR 1A [26]. Consequently, AMACR has attracted considerable interest as a prostate cancer biomarker [1,2,27] and drug target [25,28–31]. However, the lack of a convenient assay to measure AMACR activity [32,33] has severely hampered the development of AMACR inhibitors as new chemotherapeutic drugs against cancers that over-express AMACR, and consequently only a few rationally designed inhibitors of AMACR [28–30,34] or MCR [31,35] (*M. tuberculosis* homologue) have been reported. No systematic study of AMACR

Abbreviations: AMACR, α-methylacyl-CoA racemase; CDI, carbonyldiimidazole; DAST, (Diethylamino)sulfur trifluoride; DCC, Dicyclohexylcarbodiimide; DMAP, (dimethylamino) pyridine; MCR, 2-methylacyl-CoA racemase from *M. tuberculosis*; SAR, Structure-activity relationships; THF, tetrahydrofuran; TMSCl, chlorotrimethylsilane

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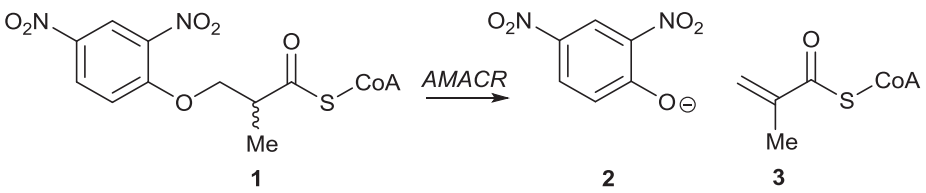

Scheme 1. The colorimetric assay for AMACR 1A [32] showing elimination of 2,4-dinitrophenolate **2**.

inhibitor SAR has been undertaken [32,33].

Recently, we reported a versatile continuous assay for AMACR based on the utilisation of our novel substrate **1** that can eliminate 2,4-dinitrophenolate **2**, which can be monitored by absorbance at 354 nm, and unsaturated product **3** (Scheme 1). This new assay [32] was used to examine the potency of two known acyl-CoA inhibitors (*N*-dodecyl-*N*-methylcarbamoyl-CoA **4** [29] and ibuprofenoyl-CoA **5** [6]; Fig. 1) and selected known non-specific protein-modification agents [25]. This paper reports the first systematic examination of SAR for rationally designed acyl-CoA inhibitors of AMACR. Compounds investigated (Fig. 1) include those with aromatic side-chains, (**5–11**); Straight-chain acyl-CoA esters (**12–17**); Branched-chain substrates (**18–21**) and product **22**. Analogues of known inhibitors with modified 2-methylacyl-CoA moieties (**4**, **23–26**) were also examined. The results reveal a correlation between potency and lipophilicity of the inhibitors, consistent with observations on MCR inhibitors [35], the homologous enzyme from *M. tuberculosis*.

2. Results and discussion

AMACR is a promising novel cancer drug target, but therapeutic development in this field has been slow due to the lack of a robust enzyme assay. Thus, the majority of studies reporting AMACR inhibitors have largely focussed on rationally designed drugs [28–30,32,33]. In most cases, only one or a few examples of each inhibitor type has been evaluated, and no systematic SAR study has been performed. Initial SAR studies have been carried out on reversible [31] and irreversible [35] inhibitors of MCR (the *M. tuberculosis* homologue). In addition, different research groups have used different assays during their studies, making it difficult to compare results directly. In this study, the SAR of rational AMACR inhibitors were explored using a series of acyl-CoA esters (Fig. 1). These included compounds previously tested as substrates (**5–11** [6,11]; **12–17** [36]; **18–20**, **22** [7,10]). Most of these compounds have not been tested as inhibitors with the exception of **5** [28,32,37,38] and **13**, **15–17** (which were previously reported to be inactive [37,38]). Compound **21** was included as an epimer of **20**, and has not been previously reported as a substrate or inhibitor (although the 3-fluoro-2-methylhexadecanoyl-CoA analogues are potent inhibitors [28]). Compound **24** is a synthetic intermediate to **25**, and has not been previously tested as a substrate or inhibitor. Compounds **22**, **25** and **26** are intermediates in the subsequent β-oxidation pathway [39], and have not been previously tested as substrates or inhibitors. Analogues of compounds **23**, and **25** with different side-chains have been previously tested as inhibitors of AMACR or MCR [9,30]. Compound **4** was previously reported as the most potent AMACR inhibitor [29,32], and is included as an acyl-CoA core analogue.

2.1. Chemical synthesis of acyl-CoA inhibitors

(2*S*,3*S*)-3-Fluoro-2-methyldecanoyl-CoA **21** was synthesised by an analogous route to (2*R*,3*R*)-3-fluoro-2-methyldecanoyl-CoA **20** [10,28], using an Evans' auxiliary strategy (Scheme 2). Aldol-like reaction of deprotonated **27** with octanal gave the (2*S*,3*R*)-3-hydroxy-2-methyl intermediate **28**. From here, alcohol **28** was activated and replaced with fluoride with inversion of configuration, using DAST to give 3-fluoro-2-methyl derivative **29**. The reaction is thought to go with inversion of stereochemical configuration (by analogy with the work of Carnell *et al.* [28]). Removal of the Evans' auxiliary from intermediate **29** provided the carboxylic acid **30** under mild conditions that involved *in situ* generation of lithium hydroperoxide. Intermediate **30** was subsequently converted to the CoA thioester **21** using the standard synthetic method with *N*,*N*'-carbonyldiimidazole [6,10,11,32,33,40]. This compound was stable in solution in the absence of AMACR, showing that the relative geometry of the α-proton and fluorine atom was *syn*- (*anti*- epimers rapidly eliminate fluoride, presumably by an E2 mechanism [10]).

2-Methylenedecanoyl-CoA **23** was synthesised by an adaptation of the method reported by Morgenroth *et al.* [30] (Scheme 3). Meldrum's acid **33** was acylated with octanoic acid **32** using DCC activation; the intermediate ketone was reduced to the octyl-Meldrum's acid **31** with sodium triacetoxyborohydride generated *in situ*. Subsequent reaction of **31** with Eschenmoser's salt gave the 2-methylene ester **34**. Base-hydrolysis furnished the 2-methylene acid **35**, which was coupled with CoA-SH by a mixed anhydride approach to give 2-methylenedecanoyl-CoA **23**.

2-Methyl-3-oxodecanoyl-CoA **25** was synthesised by the method of Reen *et al.* [41] from **36** (Scheme 4). The ketone in **37** was protected as the cyclic acetal **36**. Hydrolysis of the ester group in **36** gave the corresponding acid **38**, which was then coupled with CoA [6,10,11,32,33,40] to give **24**. Acidolysis of the acetal protection provided **25**.

2*S*,3*R*-3-Hydroxy-2-methyldecanoyl-CoA **26** was synthesised (Scheme 5) from the acyl-Evan's auxiliary **28** (Scheme 2, *vide supra*) by hydrolysis with lithium hydroperoxide to give acid **39**, which was converted to the CoA ester **26** using the standard procedure [6,10,11,32,33,40].

2.2. Evaluation of inhibitors

The selected AMACR inhibitors were evaluated using the colorimetric assay [32]. Incubation of active human AMACR 1A with substrate **1** results in production of 2,4-dinitrophenolate **2** and unsaturated product **3** (Scheme 1). Hence, enzyme activity can be determined based on measuring the absorbance of **2** at 354 nm over the assay time course. Inhibitory potency was assessed using dose–response curves to determine IC_{50} values (Fig. 1).

As expected, ibuprofenoyl-CoA **5** and its derivatives **6–9** were inhibitors of the enzyme, with most having IC_{50} values of *ca.* 500 nM (Fig. 1). Variation of the structure of the side-chain in these inhibitors appeared to make little difference to inhibitory activity (as judged by IC_{50} values), although fenoprofenoyl-CoA **6** appeared to be slightly more potent than the other examples and naproxenoyl-CoA **9** appeared to be slightly less so. All these compounds are known substrates of AMACR [6] and are predicted to behave as competitive inhibitors. Ibuprofenoyl-CoA **5** has been previously confirmed to be a competitive inhibitor of AMACR, with K_i = 60 nM [32], consistent with observations of other workers on the human and rat enzymes [28,37,38]. The mandelic acid derivatives *R*- and *S*-2-hydroxy-2-phenylacetyl-CoA **10** and **11** were also modest inhibitors (Fig. 1), binding approximately ten times less strongly than compounds **5–9**. Compounds **10** and **11** are not substrates of AMACR, since enzyme-catalysed α-proton exchange does not occur [11]. This result with **10** and **11** also demonstrates that inhibitors can possess a 2-hydroxy- group in addition to the previously