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Identification of highly potent N-acylethanolamine acid amidase (NAAA) inhibitors: Optimization of the terminal phenyl moiety of oxazolidone derivatives

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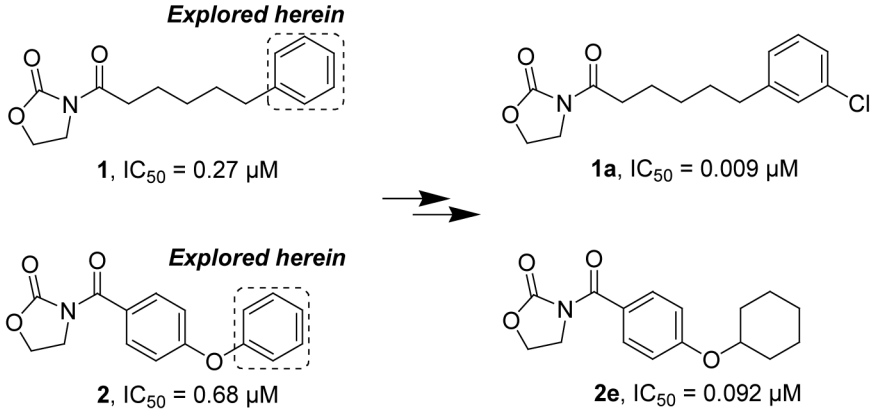
Graphical abstract



Highlights

Structure-activity relationship studies on oxazolidone derivatives to identify the structural requirements for inhibition of N-acylethanolamine acid amidase (NAAA) .

Selectivity and inhibition mechanism studies on the newly synthesized NAAA inhibitors based on oxazolidone derivatives.

Discovery of **1a** (F215) as a highly selective and nanomolar potent NAAA inhibitor.

Abstract

N-acylethanolamine acid amidase (NAAA) is a cysteine hydrolase that participates in the deactivation of fatty acid ethanolamides, such as palmitoylethanolamide (PEA). NAAA inhibition may provide a potential therapeutic strategy for the treatment of diseases in which higher PEA level is desired. In the present study, we reported the structure-activity relationship (SAR) studies for oxazolidone derivatives as NAAA inhibitors. A series of substituents or alkyl replacements for the terminal phenyl ring of oxazolidone derivatives were examined. The results showed that the inhibition potency of these oxazolidone derivatives towards NAAA depends on the sizes, flexibility, and lipophilicity of the terminal groups. SAR results suggested that small lipophilic 3-phenyl substituents or hydroxy-containing 4-phenyl substituents were preferable for optimal potency. Furthermore, the distal aliphatic replacement is also preferred for high inhibitory potency. Rapid dilution and kinetic analysis suggested that oxazolidone derivatives with different terminal phenyl moieties inhibited NAAA via different mechanisms. This study identified several highly potent NAAA inhibitors, including **1a** (F215, IC_{50} = 0.009 μM), **1o** (IC_{50} = 0.061 μM) and **2e** (IC_{50} = 0.092 μM), and also determined structural requirements of oxazolidone derivatives for potent inhibition against NAAA.

Keywords

N-acylethanolamine acid amidase (NAAA), palmitoylethanolamide (PEA), NAAA inhibitor, oxazolidone derivatives, structure-activity relationship (SAR)

1. Introduction

N-acylethanolamine acid amidase (NAAA), a cysteine hydrolase enzyme widely expressed in immune cells, e.g., macrophages and B-lymphocytes, is one of the key enzymes in the degradation of fatty acid ethanolamides, especially for palmitoylethanolamide (PEA) [1-3]. PEA involves in various physiological processes, such as the regulation of pain, inflammation and cell degranulation, through the activation of nuclear peroxisome proliferator-activated receptor α (PPAR-α) [4-7]. Pharmacological blocking of NAAA increases endogenous PEA levels in rodent models, inducing analgesic and anti-inflammatory effects [8, 9]. Inhibition of NAAA showed no undesirable cardiovascular effects and gastrointestinal haemorrhaging as seen with Cyclo-oxygenase-2 (COX-2) inhibitors [10, 11]. NAAA inhibitors, therefore, provide a potential therapeutic strategy for the treatment of pain, inflammatory and other related diseases [12].

Although development of potent NAAA inhibitors has attracted increasing interest recently, only a few potent NAAA inhibitors have been identified so far [12-14]. Most of the recently reported potent inhibitors are the reactive β-lactone/β-lactam derivatives, including ARN077 and ARN726, which can form a covalent bond with the cysteine nucleophile of NAAA, thereby inactivating NAAA [8, 15-19]. However, inhibitors with such structure show low chemical and plasma stability, thus limiting their therapeutic applications [18]. Another category of NAAA inhibitors are oxazolidone derivatives, such as **1** (F96) and **2** [10, 11]. Inhibitors with such structure are potent and selective towards NAAA inhibition, and are also systemically active and exhibited low toxicity in animal models [10, 11]. *In vivo* studies showed that compound **1** effectively normalized PEA levels in inflamed tissues. The profound analgesic and anti-inflammatory effects was seemed in several animal models, including acetic acid-induced visceral pain, formalin-induced inflammatory pain, sciatic nerve injury (SNI)-induced neuropathic pain, and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema models [10, 11].

We recently reported a structure-activity relationship (SAR) study targeting the oxazolidone ring and the link chain of **1**, and disclosed several selective and potent NAAA inhibitors [11]. In the present study (Figure 1), we further modified the terminal phenyl group of **1** and **2** to develop more potent NAAA inhibitors and to better understand the structure-activity relationship of oxazolidone derivatives. We explored the sizes, flexibility, and lipophilic requirements of the terminal moiety for potent NAAA inhibition, and also evaluated the impact of these structural features on the inhibition mechanism. These studies allowed us to identify a series of highly potent NAAA inhibitors, including **1a** (IC_{50} = 0.009 μM), **1o** (IC_{50} = 0.061 μM) and **2e** (IC_{50} = 0.092 μM), and disclosed the structural requirements of the terminal moiety of oxazolidone derivatives for potent NAAA inhibition.

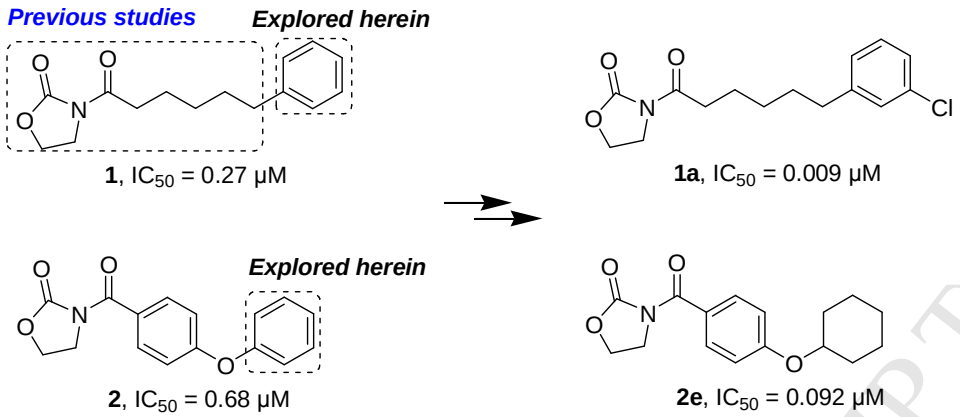


Figure 1. Design strategy for oxazolidone derivatives as NAAA inhibitors.

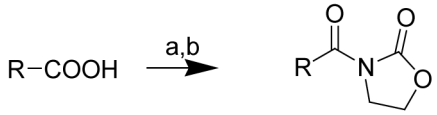
2. Chemistry

Oxazolidone imides **1a-1d**, **1f**, **1k-1n**, **1p**, **1aa**, **1bb**, **2a**, **2c**, **2d-2i** and **2k** were prepared by imidation of the appropriate acyl chloride with 2-oxazolidone. The acyl chlorides were obtained by reaction of corresponding acids with oxalyl dichloride (Scheme 1) [11]. Acids **1a-1~1d-1**, **1f-1**, **1k-1~1n-1**, **1p-1**, **1aa-1**, **1bb-1** were synthesized by a catalytic hydrogenation of the respective intermediates, prepared by the Wittig reaction in the presence of lithium hexamethyldisylazane (LHMDS) (Scheme 2) [20]. Acids **2a-1**, **2c-1** and **2d-1** were prepared by copper-mediated cross-coupling reactions with methyl 4-hydroxybenzoate and phenylboronic acids, and subsequent hydrolysis of corresponding esters (Scheme 3) [21]. Acids **2e-1**~**2i-1** and **2k-1** were obtained by reactions of methyl 4-hydroxybenzoate with alcohols and subsequent hydrolysis of esters (Scheme 4) [21]. Oxazolidone imides **1e**, **1g**, **1h**, **1o**, **1q** and **1r** were prepared by catalytic hydrogenation of the O-protected intermediates, obtained by the reactions of acids **1e-1**, **1g-1**, **1h-2**, **1o-1**, **1q-1**, **1r-2** with 2-oxazolidone (Scheme 5). Acids **1e-1**, **1g-1**, **1o-1**, **1q-1** were synthesized by Wittig reaction of the phosphonium salt and corresponding O-protected aldehyde (Scheme 6) [20]. Acids **1h-2** and **1r-2** were synthesized by the Wittig reactions of commercially unavailable aldehyde **1h-1** and **1r-1**, generated by a palladium catalyzed arylation of allylic alcohol with iodobenzene (Scheme 7) [22]. Compounds **1i** and **1s** were obtained by reaction of **1h** and **1r** with oxalyl dichloride, respectively, followed by treatment with ammonium bicarbonate (NH_4HCO_3) in acetonitrile (CH_3CN) (Scheme 8). Compounds **1j** and **1t** were obtained by the Curtius rearrangement of appropriate acylazide, prepared by the **1h** and **1r** with diphenylphosphorylazide (DPPA), respectively (Scheme 9) [20].

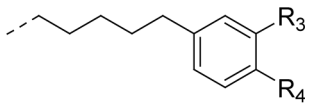
3. Enzyme assay

The NAAA inhibition activity of newly synthesized compounds was tested using heptadecenoylethanolamide (C17:1 FAE) as substrate, and the residual hydrolysis products of the substrate were determined by liquid chromatography-mass spectrometer (LC-MS). NAAA inhibitor (*S*)-OOPP (10 μM) was used as the positive

control. All statistical analysis was performed using the Graphpad Prism 5 software package, and IC_{50} values were calculated from concentration response curves using nonlinear regression analysis. All experiments were performed in triplicate (n = 3).

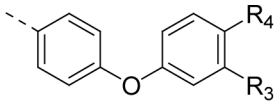


1a-1d, 1f, 1k-1n, 1p, 1aa, 1bb, 2a, 2c-2k

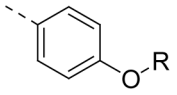


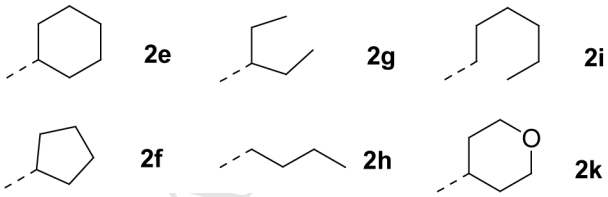
R_4 = H, R_3 = Cl	**1a**	R_3 = H, R_4 = Cl	**1k**
R_4 = H, R_3 = Br	**1b**	R_3 = H, R_4 = Br	**1l**
R_4 = H, R_3 = I	**1c**	R_3 = H, R_4 = I	**1m**
R_4 = H, R_3 = SMe	**1d**	R_3 = H, R_4 = SMe	**1n**
R_4 = H, R_3 = OMe	**1f**	R_3 = H, R_4 = OMe	**1p**
		R_3 = CH_3, R_4 = CH_3	**1aa**
		R_3 = Cl, R_4 = F	**1bb**

R =

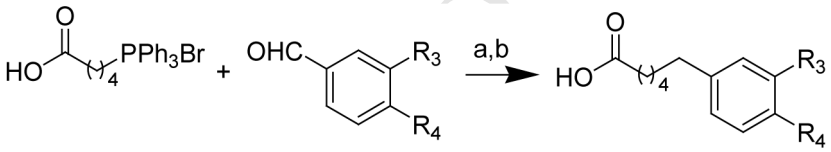


R_3 = Cl, R_4 = H	**2a**
R_3 = Me, R_4 = H	**2c**
R_3 = H, R_4 = Me	**2d**





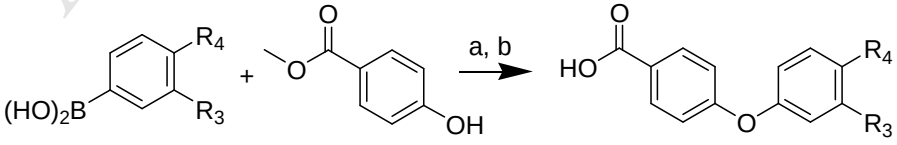
Scheme 1. Synthesis of compounds **1a-1d**, **1f**, **1k-1n**, **1p**, **1aa**, **1bb**, **2a**, **2c-2k**. Reagents and conditions: (a) oxalyl dichloride, cat. DMF, CH_2Cl_2, 0°C-rt, 1 h; (b) n-BuLi, 2-oxazolidone, THF, −78°C-rt, 4 h.



1a-1~1d-1, **1f-1**, **1k-1~1n-1**, **1p-1**, **1aa-1**, **1bb-1**

R_4 = H, R_3 = Cl	**1a-1**	R_3 = H, R_4 = Cl	**1k-1**	R_3 = CH_3, R_4 = CH_3	**1aa-1**
R_4 = H, R_3 = Br	**1b-1**	R_3 = H, R_4 = Br	**1l-1**	R_3 = Cl, R_4 = F	**1bb-1**
R_4 = H, R_3 = I	**1c-1**	R_3 = H, R_4 = I	**1m-1**		
R_4 = H, R_3 = SMe	**1d-1**	R_3 = H, R_4 = SMe	**1n-1**		
R_4 = H, R_3 = OMe	**1f-1**	R_3 = H, R_4 = OMe	**1p-1**		

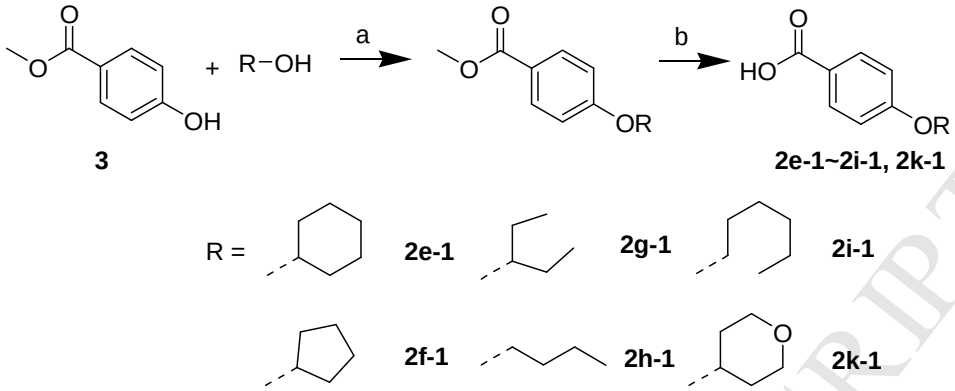
Scheme 2. Synthesis of compounds **1a-1~1d-1**, **1f-1**, **1k-1~1n-1**, **1p-1**, **1aa-1**, **1bb-1**. Reagents and conditions: (a) LHMDS, THF, −78°C-room temperature (rt), overnight; (b) H_2, Pd/C, CH_3OH, rt, 2 h.



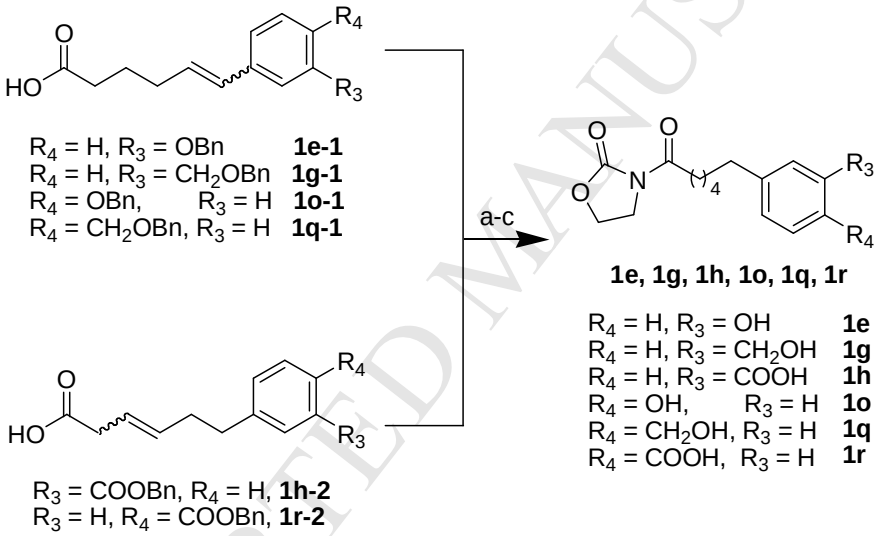
2a-1, 2c-1, 2d-1

R_3 = Cl, R_4 = H,	**2a-1**
R_3 = Me, R_4 = H,	**2c-1**
R_3 = H, R_4 = Me,	**2d-1**

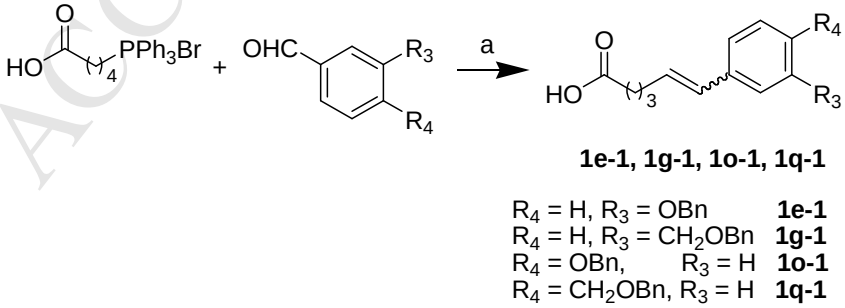
Scheme 3. Synthesis of compounds **2a-1**, **2c-1** and **2d-1**. Reagents and conditions: (a) $Cu(OAc)_2$, pyridine, 4 Å molecular sieves; (b) NaOH, $EtOH/H_2O$, then 3N HCl



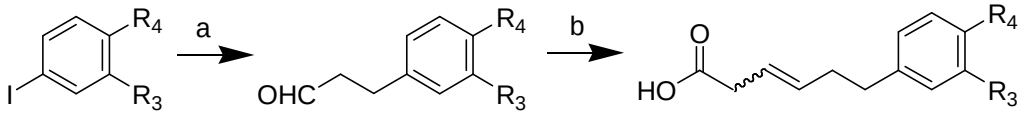
Scheme 4. Synthesis of compounds **2e-1~2i-1** and **2k-1**. Reagents and conditions: (a) DIAD, PPh_3, THF, 0°C-rt, 4 h; (b) NaOH, $EtOH/H_2O$, then 3N HCl.



Scheme 5. Synthesis of compounds **1e**, **1g**, **1h**, **1o**, **1q** and **1r**. Reagents and conditions: (a) oxalyl dichloride, cat.DMF, CH_2Cl_2, 0°C-rt, 1 h; (b) n-BuLi, 2-oxazolidone, THF, −78°C-rt, 4 h; (c) H_2, Pd/C, CH_3OH, rt, 2 h



Scheme 6. Synthesis of compounds **1e-1**, **1g-1**, **1o-1** and **1q-1**. Reagents and conditions: (a) LHMDS, THF, −78°C-rt, overnight;

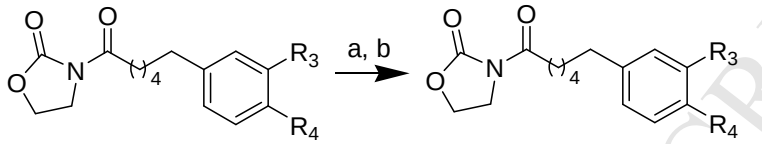


1h-1, 1r-1 **1h-2, 1r-2**

R_3 = COOBn, R_4 = H, **1h-1**
R_3 = H, R_4 = COOBn, **1r-1**

R_3 = COOBn, R_4 = H, **1h-2**
R_3 = H, R_4 = COOBn, **1r-2**

Scheme 7. Synthesis of compounds **1h-2** and **1r-2**. Reagents and conditions: (a) allyl alcohol, $Pd(OAC)_2$, TBACl, $NaHCO_3$, DMF, 50°C; (b) $BrPh_3PCH_2CH_2COOH$, LHMDS, THF, −78°C-rt, overnight

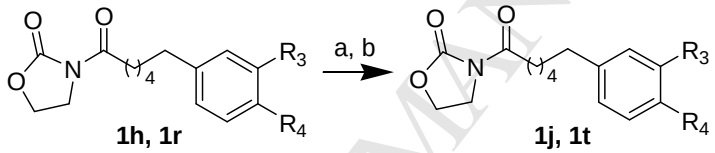


1h, 1r **1i, 1s**

R_4 = H, R_3 = COOH **1h**
R_4 = COOH, R_3 = H **1r**

R_4 = H, R_3 = $CONH_2$ **1i**
R_4 = $CONH_2$, R_3 = H **1s**

Scheme 8. Synthesis of compounds **1i** and **1s**. Reagents and conditions: (a) oxalyl dichloride, CH_2Cl_2, rt, 1 h; (b) NH_4HCO_3, rt, 2 h.



1h, 1r **1j, 1t**

R_4 = H, R_3 = COOH **1h**
R_4 = COOH, R_3 = H **1r**

R_4 = H, R_3 = NH_2 **1j**
R_4 = NH_2, R_3 = H **1t**

Scheme 9. Synthesis of compounds **1j** and **1t**. Reagents and conditions: (a) DPPA, Et_3N, toluene, rt, 30 min; (b) reflux, 30 min.

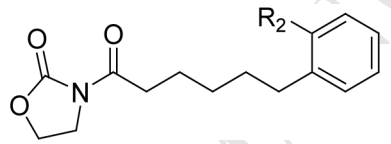
4. Results and discussion

4.1 Substitution of the terminal phenyl ring of 1

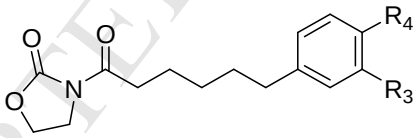
In preliminary SAR study, a series of phenyl substitutions for the terminal phenyl ring of **1** were evaluated. (Table 1, Table S1). Substitution at the position 2 with either lipophilic or polar groups (2-CH_3, **3**; 2-Cl, **4**; or 2-OH, **5**) reduced inhibitory potency compared to the unsubstituted phenyl derivative **1**, indicating that position 2 is unsuitable for further modification. Substitution at the position 3 with lipophilic groups afforded effective inhibitors (**1a-1d**), while polar substituents greatly diminished the potency (**1e-1j**). The potency of these derivatives was dependent on the size of the 3-substituent. 3-Cl (**1a**) and 3-CF_3 (**1u**) significantly enhanced inhibitory activity, while 3-Br (**1b**) and 3-CH_3 (**1v**) matched the activity of **1**. Bulky substituents (3-I, **1c**; 3-SCH_3, **1d**) dramatically decreased NAAA inhibition potency. In contrast, the 4-substituents showed different patterns of potency changes. Lipophilic functionality (**1k-1n**, **1p**, **1w**) was poorly tolerated in position 4, while polar hydroxyl-containing substituents (4-OH, **1o**; 4-CH_2OH, **1q**) significantly enhanced the potency. However, much more polar (4-COOH, **1r**; 4-$CONH_2$, **1s**) and

basic (4-NH_2, **1t**) groups substantially decreased the potency. 4-CH_3 substituted **1x** (IC_{50} = 0.44 μM) was less potent than hydroxyl-containing analogs (**1o**, IC_{50} = 0.061 μM and **1q**, IC_{50} = 0.19 μM). We speculated that 4- substitution sits in a small hydrophilic niche in the protein, which prefers polar hydroxyl (**1o** and **1q**) to lipophilic methyl (**1x**). Previous studies suggested that the acyl chain of oxazolidone derivatives occupies a hydrophobic pocket of NAAA that binds the fatty acyl chain of substrates, and incorporation of polar moieties into the acyl side chain substantially decreased the binding affinity [11, 23]. We are surprised to find that the polar hydroxyl can be well tolerated in the hydrophobic pocket. The preliminary SAR study showed that introduction of small lipophilic groups in the position 3 or hydroxyl-containing substituents in the position 4 significantly improved inhibitory potency. Several oxazolidone derivatives were identified as nanomolar potent NAAA inhibitors, e.g., **1a** (IC_{50} = 0.009 μM) and **1o** (IC_{50} = 0.061 μM).

Table 1. Inhibitory effects of compounds **3-5**, **1a-1x**, **1aa-1dd** on rat NAAA activities. Data are presented as mean ± standard deviation (SD) of three independent experiments mean. All experiments were performed in triplicate (n = 3).



	R_2	IC_{50} ± SD (μM)		R_2	IC_{50} ± SD (μM)
3	-CH_3	> 20	**5**	-OH	> 20
4	-Cl	> 20			



	R_3	R_4	IC_{50} ± SD (μM)		R_3	R_4	IC_{50} ± SD (μM)
1a	-Cl		0.009 ± 0.001	**1k**		-Cl	4.60 ± 0.83
1b	-Br		0.43 ± 0.02	**1l**		-Br	8.51 ± 2.90
1c	-I		3.12 ± 0.98	**1m**		-I	> 20
1d	-SCH_3		12.8 ± 4.01	**1n**		-SCH_3	> 20
1e	-OH		8.64 ± 1.25	**1o**		-OH	0.061 ± 0.015
1f	-OCH_3	-H	16.30 ± 5.80	**1p**	-H	-OCH_3	> 20
1g	-CH_2OH		> 20	**1q**		-CH_2OH	0.19 ± 0.04
1h	-COOH		> 20	**1r**		-COOH	> 20
1i	-$CONH_2$		> 20	**1s**		-$CONH_2$	> 20
1j	-NH_2		> 20	**1t**		-NH_2	15.70 ± 6.20
1u	-CF_3		0.058 ± 0.012	**1w**		-CF_3	1.04 ± 0.23

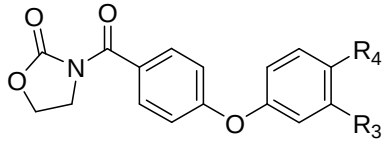
1v	$-CH_3$		0.35 ± 0.05	**1x**		$-CH_3$	0.44 ± 0.06
1aa	-Me	-Me	> 20	**1cc**	-Cl	-OH	> 20
1bb	-Cl	-F	0.026 ± 0.004	**1dd**	-F	-OH	0.16 ± 0.04

Next, we tested whether di-substitution of the terminal phenyl group of **1** by highly preferable 3- and 4-substituents can enhance the potency (Table 1). Unfortunately, the NAAA inhibition potency of di-substituted compounds **1aa** (3-CH_3, 4-CH_3) and **1cc** (3-Cl, 4-OH) was almost completely diminished compared to their mono-substituted analogues. However, additional -F can be tolerated in 3-Cl and 4-OH substituted oxazolidone derivatives (**1bb**, **1dd**), but -F modification produced 2.9- and 2.6-fold decreases in potency with respect to the mono-substituted **1a** and **1o**.

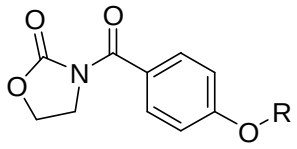
4.2 Modification of the terminal phenyl ring of 2

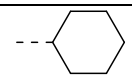
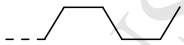
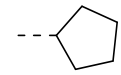
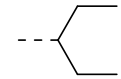
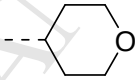
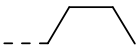
Except from the distal moieties, the flexibility of the link chain of oxazolidone derivatives also has critical influence on the NAAA inhibition potency [11]. Analogues of **1** bearing conformationally flexible hexanoyl link chain, which may favour the correct positioning of different distal moieties, thus showed high potency. We then tested whether the identified preferable distal moieties can increase the potency of oxazolidone derivatives containing conformationally constrained link chain. We then did a SAR study on compound **2**, a low micromolar NAAA inhibitor (IC_{50} = 0.68 μM) with restricted phenoxyl link chain. By modification of the terminal phenyl ring, a series of phenyl substitutions for the distal phenyl group of **2** were synthesized and tested (Table 2). Unfortunately, all 3- or 4-substituted phenyl analogues (**2a**-**2d**) were less effective than unsubstituted **2**, indicating the restricted link chain may restrict the movements of distal phenyl ring of inhibitor towards the binding site. Next, oxazolidone derivatives bearing flexible aliphatic replacement for **2** were evaluated. Compared to unsubstituted **2**, the cycloalkyl substitutions (**2e**, **2f**) were 6-fold more effective, while 3-pentenyl (**2g**) and hexyl derivatives (**2i**) were comparable potent. The results are in agreement with previous observation that replacement of the phenyl ring of **1** with aliphatic group exceeded the potency [11]. Incorporation of polar hydroxyl (**2j**) or oxygen (**2k**) into the aliphatic ring didn't affect the potency. However, butyl derivative (**2h**) resulted in nearly 2-fold decrease in inhibitory potency. It has been reported that sp2 hybridized atoms can't be well tolerated at the end of hydrophobic pocket of NAAA, thus NAAA inhibitors with such groups exhibited weak potency [24]. Replacement of the phenyl ring with the aliphatic groups may avoid the unfavourable binding occupancy of the hydrophobic pocket by the sp2 hybridized carbon, therefore enhanced the NAAA inhibition. These results confirmed the beneficial effects of the terminal aliphatic ring of oxazolidone derivatives towards NAAA inhibition, and several potent NAAA inhibitors, e.g., **2e** (IC_{50} = 0.092 μM) were identified.

Table 2. Inhibitory effects of compounds **2a-2k** on rat NAAA activities. Data are presented as mean ± standard deviation (SD). All experiments were performed in triplicate (n = 3).



	R_3	R_4	IC_{50} ± SD (μM)		R_3	R_4	IC_{50} ± SD (μM)
2	-H	-H	0.68 ± 0.11	**2c**	-Me	-H	5.21 ± 1.33
2a	-Cl	-H	7.74 ± 1.84	**2d**	-H	-Me	6.40 ± 1.57
2b	-H	-OH	> 20				



	R	IC_{50} ± SD (μM)		R	IC_{50} ± SD (μM)
2e		0.092 ± 0.028	**2i**		0.64 ± 0.18
2f		0.12 ± 0.03	**2j**		0.21 ± 0.05
2g		0.48 ± 0.10	**2k**		0.33 ± 0.08
2h		1.34 ± 0.30			

4.3 Selectivity evaluation

Acid ceramidase (AC) is another lysosomal cysteine amidase that showed 33-35% amino acid identity with NAAA. To understand the selectivity of oxazolidone derivatives towards NAAA against AC, the activity of the most active oxazolidone derivatives (**1a**, **1o** and **2e**) against h-AC was tested. As shown in Table 3, **1a**, **1o** and **2e** showed weak inhibition activity against AC. All the three representative oxazolidone derivatives reducing no more than 20% at a concentration of 20 μM, at which concentration NAAA was completely inhibited by these oxazolidone derivatives. The results suggested that oxazolidone derivatives were highly selective for NAAA over AC.

Table 3. Effects of compounds **1**, **1a**, **1o** and **2e** on rat-AC activity. Data are presented as mean ± standard deviation (SD). All experiments were performed in triplicate (n = 3).

	Vehicle	**1** (20μM)	**1a** (20μM)	**1o** (20μM)	**2e** (20μM)
IC_{50} ± SD (μM)	---	0.27 ± 0.04	0.009 ± 0.001	0.061 ± 0.015	0.092 ± 0.028
AC activity (%)	100.0 ± 21.4	90.6 ± 18.2	85.3 ± 12.5	95.1 ± 17.4	81.7 ± 9.4

4.4 Mechanistic study

Oxazolidone derivatives share similar electrophilic structures with β-lactone derivatives, one type of NAAA inhibitors that are known to interact covalently with the active site of NAAA. Therefore, we postulated that oxazolidone derivatives may inactivate NAAA via similar mechanisms as β-lactone. To demonstrate this hypothesis, we investigated the interaction mechanism of **1a** with NAAA by a Yonetani-Theorell analysis, an approach widely used to discriminate whether two inhibitors potentially binding to the same or overlapping site [25-26]. **1a** was subsequently tested in combination with ARN077 [16], a well known covalent β-lactone NAAA inhibitor, or 3-biphenylpropaonoyl pyrrolidine [13], a competitive inhibitor that binds non-covalently in the same active site of NAAA as ARN077. Results showed a series of parallel lines, indicating that **1a** bound to NAAA in a mutually exclusive fashion with ARN077 (Figure. 2A) and 3-biphenylpropaonoyl pyrrolidine (Figure. S1). These data suggested that **1a** binds to the active site of NAAA similar to β-lactone derivative. Further kinetic analysis showed that **1a** decreased the maximal catalytic velocity (Vmax) of NAAA, but did not affect Michaelis-Menten constant Km (Figure. 2B), suggesting a non-competitive and probably covalently inhibition mechanism. Moreover, rapid dilution revealed that **1a** inhibited NAAA via a slowly reversible mechanism (Figure. 2C). When combined, these results suggested that oxazolidone derivatives may form a covalent yet reversible bond with NAAA, thus inhibited the enzyme activity.

However, the enzyme kinetics data showed that **1k**, the 4-regioisomer of **1a**, was a weak, competitive and rapidly reversible NAAA inhibitor (Figure 2B, 2C, Table 4). There may be two reasons for the different inhibition mechanism and potency. First, the 4-Cl decreases the binding affinity of the inhibitors, preventing the formation of the NAAA-inhibitor complex, thus resulting in poor potency and rapidly reversible binding. Second, the 4-Cl enhances the degradation of NAAA-inhibitor complex compared to 3-Cl, and inhibitors containing 4-Cl are rapidly converted to other non-active structures. Therefore, the degradation of **1a** and **1k** by NAAA was studied to test the possible mechanisms. The results showed that compound **1a** was slowly cleaved by NAAA (Figure 2D), and there was 92.6% of **1a** remaining after 6 h incubation, suggesting that **1a** was covalently attacked by NAAA, and slowly converting to other compounds. However, for weak inhibitor **1k**, no significant degradation was observed when tested under the same conditions. The results suggested that the different inhibition mechanisms of **1a** and **1k** wasn't due to the different conversion rate. Instead, it's more possible that different group influenced the binding affinity of inhibitors, thus affected the inhibition mechanism and potency.

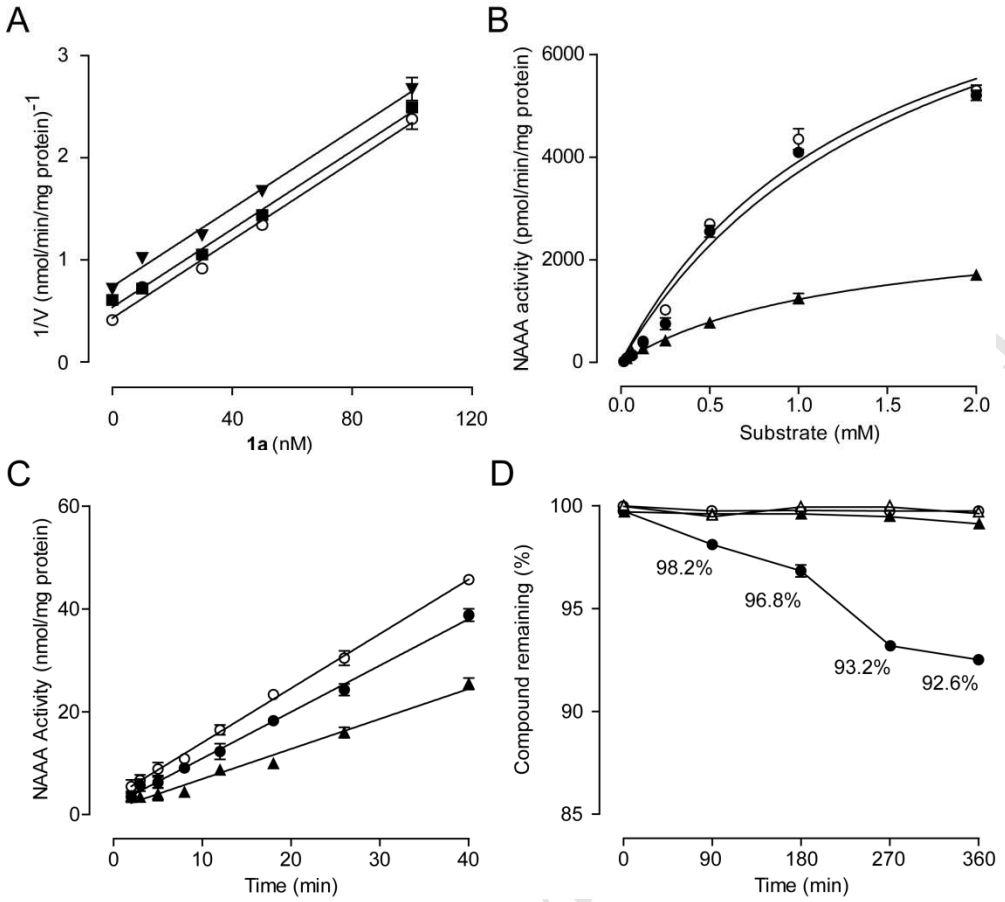


Figure 2. Characterization of the inhibition mechanisms of oxazolidone derivatives **1a** and **1k**. (A) Yonetani-Theorell plots for the inhibition of rat-NAAA by various combinations of **1a** and ARN077 (open circle, vehicle; closed square, 50 nM; closed triangle, 250 nM); (B) Kinetic analysis of rat NAAA inhibition by vehicle (open circle, 1% DMSO), **1a** (black circle, 0.05 μM) and **1k** (black triangle, 10 μM); (C) Rapid dilution assay of NAAA in the presence of vehicle (open circle), **1a** (black circle) and **1k** (black triangle); (D) Degradation experiment of **1a** (circle, 10 μM) and **1k** (triangle, 10 μM) in the present of vehicle (open plot, 1% DMSO) or NAAA (close plot, 300 ng). Data are presented as mean ± standard deviation (SD) of the mean. All experiments were performed in triplicate (n = 3).

Table 4. Michaelis–Menten analysis of compounds **1a** and **1k**

	Vmax (nmol/min/mg protein)	Km (μM)
Vehicle	9383 ± 937	1395 ± 260
1a	2793 ± 236	1278 ± 207
1k	9905 ± 1147	1671 ± 340

5. Conclusion

Here, we further optimized the terminal phenyl moiety of oxazolidone derivatives **1** and **2** to develop more potent NAAA inhibitors and explored the structural requirements for potent NAAA inhibition. SAR studies suggested that substitution of the terminal phenyl ring of **1** with small lipophilic groups at position 3, or

hydroxyl-containing substituents at position 4 significantly improved the inhibitory potency. Replacement of the terminal phenyl group of **2** with aliphatic groups also effectively enhanced the NAAA inhibition activity. Rapid dilution and kinetic analysis suggested that oxazolidone derivatives with different structures inhibited NAAA via different mechanisms. **1a** (F215) inhibited NAAA through a non-competitive and slowly reversible mechanism, while **1k** inhibited NAAA in a competitive and rapidly reversible pattern. In brief, current exploration of the sizes, flexibility, and lipophilicity of the terminal groups allowed us to define the structural requirements for potent NAAA inhibition. **1a** represents a useful probe for investigating the biological function of NAAA in inflammation and analgesia, and a promising anti-inflammatory and analgesic drug candidates.

6. Author contributions

YL and YZ synthesised compounds; QC performed NMR and LC-MS assays; LY, YtL and JR performed enzymatic assays; YL wrote the paper; YL, CL, and YQ conceived and designed the experiments.

7. Acknowledgements

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