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Research Paper

Synthesis and pre-clinical studies of new amino-acid ester thiazolide prodrugs

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ABSTRACT

Thiazolides are polypharmacology agents with at least three mechanisms of action against a broad spectrum of parasites, bacteria and viruses. In respiratory viruses they inhibit the replication of *orthomyxoviridae* and *paramyxoviridae* at a post-translational level. Nitazoxanide **1a**, the prototype thiazolide, was originally developed as an antiparasitic agent and later repurposed for the treatment of viral respiratory infections. The second generation thiazolides following nitazoxanide, such as the 5-chloro analogue RM-5038 **2a**, are also broad-spectrum antiviral agents as we have reported. Both **1a** and its effective circulating metabolite, tizoxanide **1b**, are 5-nitrothiazole derivatives, while RM-5038 **2a** and its de-acetyl derivative RM-4848 **2b** are the corresponding 5-chloro derivatives. Recently **1a** has completed phase II-III clinical trials in the United States, Canada, Australia and New Zealand in a total of 2865 adults and adolescents of at least 12 months of age with viral acute respiratory illness. Since its biodisposition is primarily seen in the gastro-intestinal tract, its efficacy in systemic viral diseases requires relatively high oral doses. The chemical synthesis of new derivatives with a better systemic absorption was therefore urgently needed. In order to improve their systemic absorption, new amino-ester prodrug derivatives of **1b** and RM4848 **2b** were prepared and tested for their animal pharmacology, pharmacokinetics and toxicology. RM-5061 **8a** in rats showed 7-fold higher blood concentration compared to **1a**: absolute bioavailability increased from 3 to 20%, with a good safety profile in animal safety pharmacology and toxicology.

1. Introduction

Nitazoxanide **1a**, the first of thiazolides discovered in 1975 by Rossignol and Cavier is a broad-spectrum antiparasitic agent effective against protozoa, nematodes, cestodes and trematodes [1]. It was registered throughout Latin America, Egypt, India and Bangladesh for the treatment of intestinal protozoa and helminths while in the United States the drug was approved for the treatment of two intestinal protozoa, *Cryptosporidium parvum* and *Giardia intestinalis* [2–4]. The antiprotozoal activity of **1a** against anaerobic organisms such as some protozoa and bacteria is due to its interference with the pyruvate ferredoxin oxidoreductase (PFOR) enzyme-dependent electron transfer reaction, which is essential to anaerobic metabolism. Against helminths and *Mycobacterium tuberculosis* it disrupts membrane potential and homeostasis of intramicro-organisms [5–8]. Thiazolides also inhibit protein disulfide isomerase (PDI) and possess a broad spectrum of activity against parasites and viruses. In *Neospora caninum*, an apicomplexan emerging protozoa related to *Cryptosporidium parvum*, **1a** binds to the NcPDI blocking the protozoan replication [9]. In respiratory viruses **1a** inhibits the replication of respiratory viruses belonging to the classes of *orthomyxoviridae* and *paramyxoviridae* at a post-translational level.[10,11]. **1a** and a number of structurally related thiazolides, e. g. RM5038 **2a** are also active against both hepatitis B and C viruses at low micromolar concentrations [12–14]. For example, against a range of respiratory viruses including H1N1 influenza A strains, **1a** exhibits IC_{50} values of 0.3–1.0 μg/mL; against a range of flaviviridae including hepatitis C,

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from 0.05 to 0.5 μg/mL; against hepatitis B (hepadnaviridae), 0.06 μg/mL.

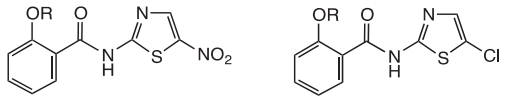
Moving to *in vivo* antiviral activity, the antiviral activity of **1a** and tizoxanide **1b**, its active circulating metabolite, was confirmed in well-controlled clinical trials carried out in more than 3000 patients in the treatment of gastroenteritis caused by rotavirus and norovirus, uncomplicated viral respiratory infections caused by influenza A and B and, alone or combined with pegylated-interferon with and without ribavirin, in the treatment of chronic hepatitis C [15–18].

1a is only partially absorbed from the gastro-intestinal tract: ^{14}C-**1a** given to human volunteers showed that 33% of the oral dose was excreted via urine and 64% was excreted in faeces. This is a perfect bio-disposition profile for a drug intended to treat intestinal pathogens, but much less desirable for a systemic antiviral agent. Upon oral absorption it is immediately metabolized into deacetyl derivative **1b**, which is subsequently metabolized in the liver as tizoxanide-glucuronide and rapidly eliminated via urine [19,20]. Ideally, the treatment of viral systemic infections as opposed to parasites or viruses infecting the intestinal tract calls for a compound with a better oral biodisposition and metabolism than **1a** but ideally liberating in the blood stream the same active circulating metabolite, **1b** and its inactive glucuronide. A considerable amount of safety and efficacy data have been accumulated for these derivatives in the United States and abroad during the last 20 years.

In summary, the thiazolides are typified by nitazoxanide **1a**, Scheme 1, and its active circulating metabolite tizoxanide **1b** as shown. Among a large number of analogues synthesised, the chloro analogue RM5038 **2a** and, similarly, its circulating metabolite RM4848 **2b** also have very good broad-spectrum antiviral activity [12–14].

Although the *O*-acetates such as **1a** are satisfactorily taken up by passive absorption, behaving effectively as ester prodrugs, the oral bioavailability of **1a** in the absence of food is typically ≤30% [20]. We therefore set out to design a robust prodrug form of **1a**/**1b**, so as to improve both the oral absorption and the solubility properties of the parent drug.

We were impressed by amino-acid based prodrug esters such as the antiviral agent valacyclovir **3**, Scheme 2 [21,22] which improves the oral bioavailability of acyclovir **4** from <20% to 54% and greatly improves its aqueous solubility; valacyclovir enters cells via the h-PEPT 1 transporter [23]. Initially, therefore, we prepared the direct analogue of tizoxanide, namely valyl ester **5** (Scheme 3). This derivative was readily prepared as the HCl salt shown [cf. Scheme 4], but unfortunately its stability proved inadequate. After 3 weeks' storage at room temperature, hydrolysis of **5** was significant, with about 20% release of the parent drug **1b** by NMR evidence.



1a R = CH_3CO **2a** R = CH_3CO

1b R = H **2b** R = H

Scheme 1. Thiazolide structures.

1.1. Chemistry

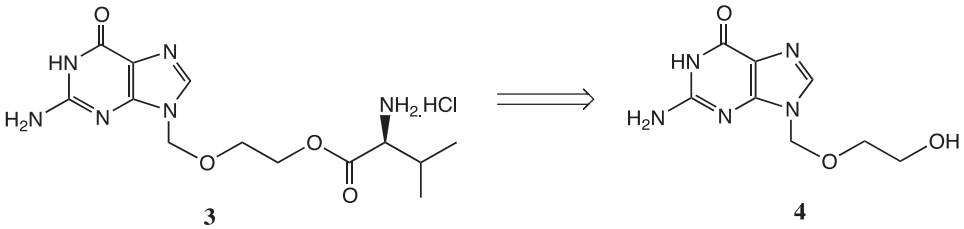
We considered that a more stable ester should result on increasing the steric bulk of the amino-acid side chain, and therefore turned next to the corresponding derivative of L-*tert*-leucine [24], Scheme 4. Reaction of **1b** with Boc-Tle-OH [25] **6** using EDC catalysed by DMAP in THF afforded protected ester **7a** in satisfactory yield after chromatography. We later found that DMF was a superior solvent, especially for **1b**, and performed well on scale-up, delivering a 90% yield of **7a.** Deprotection was very conveniently achieved by treatment of **7a** with HCl-dioxane, as the HCl salt RM5061 **8a** of the product could be crystallised directly from the reaction medium and was obtained in excellent yield. Importantly, RM5061 **8a** showed no appreciable hydrolysis after standing at 20 °C for three months (<1% of **1b** seen by NMR analysis and by HPLC); its aqueous solubility was approximately 5 mg/mL [16]. It was of paramount importance to assay the chiral purity of RM5061 **8a**; for comparison, valacyclovir is typically obtained from acyclovir in about 92% e.e. [26]. From Boc-Tle-OH (S-enantiomer), using DMF as solvent, RM5061 **8a** was obtained in 99% purity by HPLC and the e.e. was 99.8%. Starting from the corresponding derivative of D-*tert*-leucine, by chiral HPLC analysis the e. e. of *ent*-**8a** was determined to be 99.5%.

As noted above, the 5′-chlorothiazolide RM4848 **2b** is another important compound: its corresponding pro-drug was similarly made, Scheme 1. The intermediate **7b** was isolated as a crystalline solid and deprotection again proceeded smoothly to afford HCl salt RM5064 **8b** as a white solid in excellent yield. This product showed very similar stability behaviour to RM5061 **8a** (<1% hydrolysis after 3 months at 20 °C) and its aqueous solubility was greater, approximately 20 mg/mL.

The stability of valacyclovir has been extensively studied [27]. In order to probe further the issues of stability and chiral purity of products, we also prepared the isoleucine and *allo*-isoleucine derivatives **9** and **10**, Scheme 5 (see supporting information). No scrambling of NMR peaks could be seen in either product at the NMR detection level; here, epimerisation would have generated diastereoisomeric mixtures, possibly to different extents for the two products. As might have been intuitively expected, the stabilities of these compounds by NMR, ca.5% hydrolysis after 3 months at 20 °C, were greater than **5** but less than RM5061 **8a**/RM5064 **8b**.

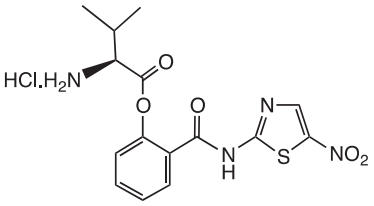
1.2. Comparative pharmacokinetics of nitazoxanide *1a*, RM-5061 *8a*, RM-5038 *2a* and RM-5064 *8b* in rats

Thiazolides are poorly soluble compounds. In rodents they are inefficiently absorbed from the gastrointestinal tract: most of the



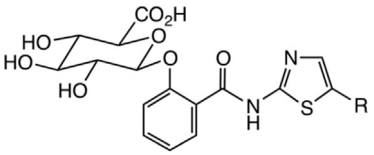
3 **4**

Scheme 2. Valacyclovir, as its HCl salt, and acyclovir.



5

Scheme 3. The L-valyl ester prodrug of tizoxanide.



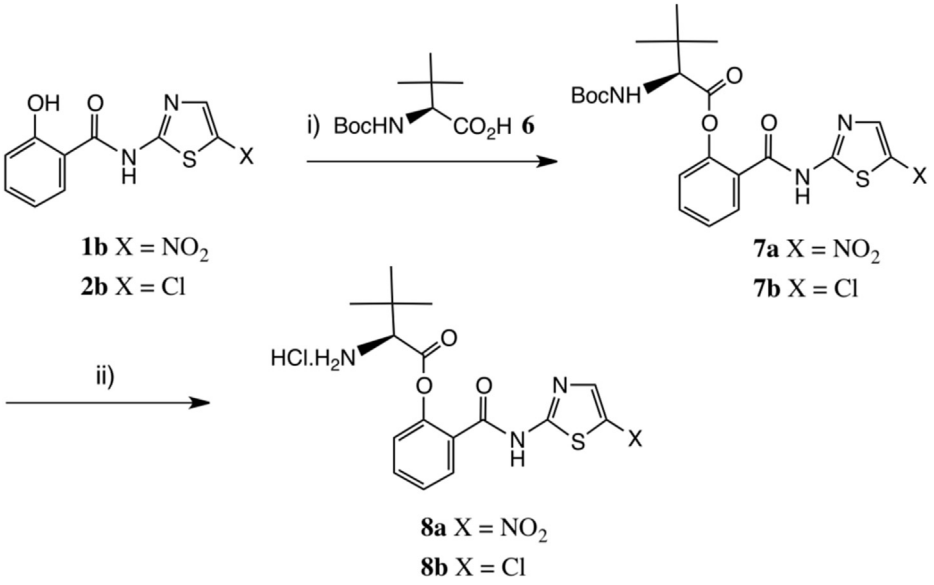
11a R = NO_2, **11b** R = Cl

Scheme 6. Thiazolide glucuronides.

compounds given orally remain in the gut. In order to assess the absolute bioavailability of our new amino-acid ester derivatives, RM-5061 **8a** and RM-5064 **8b,** in comparison with **1a** and RM5038 **2a** we carried out four parallel studies, each one including six Sprague-Dawley rats weighing about 300 g divided into two groups of three animals. In each of the four studies a group of three rats was treated with a single oral dose of each of the four compounds while the second group of three rats received a single intravenous injection of each of the same four test compounds.

For each of the four compounds the oral dose was calculated to be 30 mg/kg while the intravenous injection was 6 mg/kg, viz. 5 times less. Serial blood samples were obtained from each animal at 5, 10, 15, 30 min and 1, 2, 4, 8, 23, and 24 h post-dose. As noted above [19], tizoxanide glucuronide **11a**, Scheme 6, is the major *in vivo* metabolite of **1b** and significant concentrations of **11a** were noted after 5 min upon oral administration of either **1a** or RM-5061 **8a**; this was also the time of maximum plasma concentration.

The comparison of the AUC of **1b** calculated from the pharmacokinetics parameters obtained after oral and intravenous administrations of **1a** and RM-5061 **8a** showed a 2.8% absolute bioavailability for **1a**, 2.04 versus 70.5 after correction for the dose given to 30 mg/kg, and 20% for RM-5061 **8a**, 3.12 versus 26.0 after correction for the dose to 30 mg/kg. Interestingly, the chloro-derivative are much less bioavailable: the comparison of the AUCs of RM4848 **2b** shows essentially no oral absorption for RM5038 **2a**



1b X = NO_2
2b X = Cl

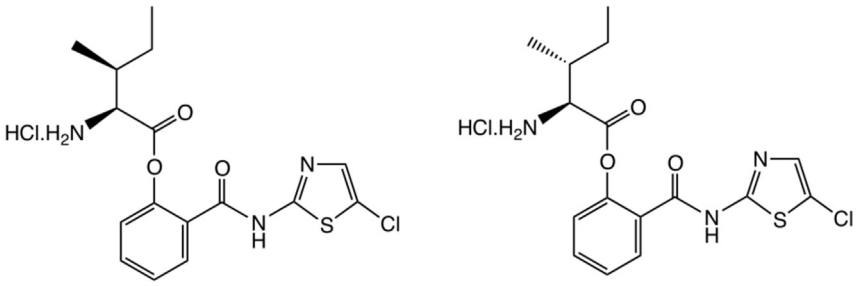
i) BocHN CO_2H **6**

ii)

7a X = NO_2
7b X = Cl

8a X = NO_2
8b X = Cl

Scheme 4. Synthesis of L-*tert*-leucyl thiazolide prodrugs. Reagents: i) Boc-Tle-OH **6**, EDC, DMAP, THF or DMF, 65%; ii) HCl-dioxane, 0–20 °C, 95%. Abbreviation: Tle = L-*tert*-leucine, (2S)- 2-amino-3,3-dimethylbutanoic acid [24].



9 **10**

Scheme 5. Isoleucyl and *allo*-isoleucyl prodrugs.

Table 1
Absolute bioavailabilities F for compounds **1a**, **8a**, **2a** and **8b**.

Compound	Bioavailability F%
Nitazoxanide **1a**	2.8
RM5038 **2a**	~0
RM5061 **8a**	20
RM5064 **8b**	22

while the amino-ester derivative, RM5064 **8b**, shows a 22% absolute bioavailability (0.23 versus 17.4 and 0.71 versus 3.15 for corrected values of the intravenous dose). This again demonstrates a better pharmacokinetic profile for the amino-acid ester derivatives than the corresponding *O*-acetyl compounds. Here again, the glucuronide metabolite **11b** [12] was quickly observed on administration of RM5038 **2a** or RM 5064 **8b** and is the main *in vivo* metabolite of RM4848 **2b**. The bioavailabilities of the four compounds are summarised in Table 1. For detailed post oral and intravenous blood levels, see supporting information.

We therefore decided to proceed with the complete pre-clinical development of RM 5061 **8a** in order to perform phase 1 human trials. Full pharmacokinetic data for all four compounds **1a**, RM 5038 **2a**, RM 5061 **8a** and RM 5064 **8b** are given in supporting information. Additionally, the new derivatives are active *in vitro* antivirals in their own right, equivalent to the parent thiazolides. Full details will be published separately.

1.3. Safety pharmacology

Two safety pharmacology studies evaluated the effect of RM-5061 **8a** on the central nervous system and on the respiratory function respectively in the rats.

Single oral doses of 100, 300 and 1000 mg/kg of RM5061 **8a** were administered to three groups of 10 rats, 5 males and 5 females, by oral gavage: one group of untreated animals was kept as a control. There were no abnormal signs recorded at 4 and 24 h after treatment at the 100 and 300 mg/kg doses but at the 1000 mg/kg dose level there was decreased activity, decreased abdominal tone, labored breathing, tremors and no pain responses observed in all animals in the group suggesting that this dose level was producing CNS toxicity but without mortality of the animals treated at this high dose.

In a second study, single oral doses of 100, 300 and 1000 mg/kg of RM5061 **8a** were given by oral gavage to three groups of six conscious rats, 3 males and 3 females to study the effects of the test drugs on respiratory function. One group of untreated animals was kept as controls. Some minor changes on the respiratory rate expressed as breaths per minute and the tidal volume were observed for the low 100 mg/kg and the 300 mg/kg oral doses without affecting the Minute Volume dose, but more pronounced effects on the three parameters recorded and described above were observed at the 1000 mg/kg dose suggesting that this level of RM5061 **8a** has some effects on the respiratory function of the rats. However, no mortality was recorded at the three dose levels tested. The dosing schedules are summarised in Table 2.

Table 2
Dosing schedules for safety pharmacology study of RM5061 **8a** in rats. One untreated group was kept as a control; for full details, see supporting information.

Indication	Grouping	Dose employed mg/kg		
CNS	3 groups, 10 rats each (5M/5F)	100	300	1000
Respiratory	3 groups, 6 rats each (3M/3F)	100	300	1000

Table 3
Dosing schedules for sub-acute toxicology study of RM5061 **8a** in rats. The doses shown were administered for 28 consecutive days; for full details, see supporting information.

Indication	Grouping	Dose employed mg/kg		
Systemic exposure to drug	4 groups, ≤ 30 rats each (15M/15F)	10	30	75

1.4. Animal toxicology of RM5061 *8a*

Two sub-acute toxicity studies of RM 5061 **8a** were carried out in Sprague-Dawley rats and Beagle dogs. The first study involved dosage of rats at three separate dose levels of 10, 30 and 75 mg/kg of RM 5061 **8a** once a day for 28 consecutive days, to evaluate systemic exposure to the drug. No terminal adverse effects were noted with the rats. Bright yellow urine was commonly observed, linked to the formulations of RM 5061 **8a**, which were yellow suspensions. Weight losses were observed, especially in the male group, but these were within acceptable limits. It was concluded that the no observed adverse effect level (NOAEL) was achieved with a dose of 10 mg/kg. Full toxicological details are given in supporting information; the dosing and group numbers are summarised in Table 3.

The second study evaluated the systemic exposure of RM-5061 **8a** when administered orally via gelatin capsule at three separate dose levels of 5, 15 and 25 mg/kg once a day for 28 consecutive days to Beagle dogs. Again, there were no early deaths with the dogs during the study period. Bright yellow urine was commonly observed and there were cases of emesis, with yellow particulate material in a few cases. However, emesis was not observed in any control animal. In both animal studies, exposure to **1b** and **11a** was apparent. Based on the overall study data, the high dose level of 25 mg/kg/day of RM 5061 **8a** administered in a single gelatin capsule for 28 consecutive days to Beagle dogs was considered a no observed adverse effect level (NOAEL). Full details are given in supporting information: the dosing and group numbers are summarised in Table 4.

2. Conclusions

Nitazoxanide **1a** was originally designed as a broad-spectrum antiparasitic drug for the treatment of intestinal protozoan and helminthic infections, for which the drug has been marketed around the world for more than 15 years. It was recently repurposed as a broad-spectrum antiviral agent in the treatment of viral acute respiratory infections. Phase III clinical trials carried out in 2865 adults and adolescents with uncomplicated influenza A and B showed that the drug reduced the duration of the influenza illness when compared to placebo with a p value < 0.05. Additionally the studies showed that nitazoxanide compared to placebo was effective in the treatment of the common cold caused by rhinovirus and coronavirus. Further studies in patients with viral respiratory infections at risk of developing complications, children,

Table 4
Dosing schedules for sub-acute toxicology study of RM5061 **8a** in beagle dogs. The doses shown were administered for 28 consecutive days; for full details, see supporting information.

Indication	Grouping	Dose employed mg/kg			
Systemic exposure to drug	4 groups, ≤10 dogs each (5M/5F)	0	10	30	75

and adults and children with severe acute respiratory infections (SARI) are currently underway. It was important to identify new derivatives with better systemic absorption, and we have now shown that RM-5061 **8a** is a second prodrug for tizoxanide **1b**. RM5061 **8a** is more soluble and better absorbed in laboratory animals than **1a** and is now undergoing Phase I clinical trials. It may provide an oral dose effective at a lower dosage, and more importantly an injectable form of tizoxanide that nitazoxanide was unable to do.

3. Experimental

3.1. Chemistry

Organic extracts were washed finally with satd. aq. NaCl and dried over anhydrous Na_2SO_4 prior to rotary evaporation at <30 °C. Analytical thin-layer chromatography was performed using Merck Kieselgel 60 F 254 silica plates. Preparative column chromatography was performed on Merck 938S silica gel. Unless otherwise stated, ^1H and ^{13}C NMR spectra were recorded on $CDCl_3$ solutions using either Bruker 250 or 400 MHz (100 MHz for ^{13}C) instruments with tetramethylsilane as internal standard. Both low- and high-resolution mass spectra were obtained by direct injection of sample solutions into a Micromass LCT mass spectrometer operated in the electrospray mode, +ve or -ve ion as indicated. CI mass spectra (NH_3) were obtained on a Fisons Instruments Trio 1000. Analytical HPLC was performed using an Ascentia Express C-18 column, eluting with a gradient of 10–100% MeCN aq.+ 0.1% v/v CF_3CO_2H and monitored at 345 nm. Chiral HPLC was performed using a Chiralpak AD-H column, eluting with *n*-C_7H_{16}: Pr^iOH, 4:1. Microanalytical data were obtained using an Elementar Vario micro cube instrument.

3.1.1. (2S)-[2-[(5-nitro-1,3-thiazol-2-yl)carbamoyl]phenyl]-2-(t-butoxycarbonyl)amino-3,3-dimethylbutanoate ***(7a)***

A mixture of Boc-Tle-OH **2** (0.21 g, 0.97 mmol) and tizoxanide **1b** (0.25 g, 0.94 mmol) was stirred at 20 °C in anhydrous THF (7.5 mL). *N*-ethyl-*N*′-3-(dimethylamino)propyl carbodiimide. HCl (EDC; 0.19 g, 1 mmol) was added in one portion, followed immediately by 4-dimethylaminopyridine (DMAP; 0.12 g, 1 mmol). After 20 h, the mixture was filtered through Celite and the precipitate washed with further THF, then diluted with ethyl acetate (25 mL). The combined filtrate and washings were washed with 7% aq. citric acid, saturated aq. $NaHCO_3$, water and brine, then dried over anhydrous Na_2SO_4. Evaporation afforded a yellow foam which was chromatographed on silica gel, being applied in CH_2Cl_2 and eluted with 1:1 ethyl acetate: hexane. Appropriate fractions were combined and evaporated to afford the title compound **7a** as an off-white solid (280 mg, 64%); ^1H NMR [400 MHz, $(CD_3)_2SO$] δ_H1.02 (9 H, s, Me_3C), 1.40 (9 H, s, Me_3CO), 4.05 (1 H, d, J = 7.6 Hz, *CH*NH), 7.25 (1H, d, J = 8.0 Hz, ArH), 7.31 (1 H, d, J = 7.6 Hz, CH*NH*), 7.47 (1 H, t, J = 8.0 Hz, ArH), 7.70 (1 H, t, J = 8.0 Hz, ArH), 7.78 (1 H, d, J = 8.0 Hz, ArH), 8.70 (1 H, s, thiazole 4-H) and 13.67 (1 H, br s, NH); ^{13}C NMR [100 MHz, $(CD_3)_2SO$] δ_C 26.9, 28.6, 34.0, 63.5, 79.0, 123.4, 126.6, 127.2, 129.9, 133.6, 142.6, 143.0, 148.4, 156.3, 162.4, 165.8 and 170.5; *m*/*z* (ES + ve mode) 501 (MNa^+, base peak). Found: *m*/*z*, 501.1417. $C_{21}H_{26}N_4O_7SNa$ requires *m*/*z*, 501.1420.

We later found that, by using DMF as solvent and 1.5 eq. of both EDC and DMAP, all reagents could be fully dissolved and a conversion of 88% of **7a** was obtained after 6 h at 0 °C; the final isolated yield was very similar.

3.1.2. (2S)-[2-[(5-chloro-1,3-thiazol-2-yl)carbamoyl]phenyl]-2-(t-butoxycarbonyl)amino-3,3-dimethylbutanoate ***(7b)***

This compound was prepared similarly to **7a**; from **2b** (0.51 g, 2 mmol) was obtained **7b** (0.62 g, 67%) as a solid which could be crystallised from EtOAc-hexane. Found: C, 53.8; H, 5.5; N, 9.1: S, 6.5. $C_{21}H_{26}ClN_3O_3S$ requires C, 53.9; H, 5.6; N, 9.0: S, 6.85%; ^1H NMR (400 MHz, $CDCl_3$) δ_H1.10 (9 H, s, Me_3CC), 1.43 (9 H, s, Me_3CO), 4.30 (1 H, d, J = 7.6 Hz, *CH*NH), 5.28 (1 H, br d, J = 7.6 Hz, CH*NH*), 6.82 (1 H, s, thiazole 4-H), 7.35–7.45 (2 H, m, 2xArH), 7.62 (1 H, t, J = 8.0 Hz, ArH), 7.89 (1 H, d, J = 8.0 Hz, ArH) and 11.66 (1 H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 26.6, 28.2, 34.4, 62.7, 80.3, 120.8, 123.2, 125.9, 126.4, 130.1, 133.1, 134.5, 148.3, 155.7, 156.6, 163.3 and 170.0; *m*/*z* (ES + ve mode) 490, 492 (MNa^+, base peaks for $^{35}Cl/^{37}Cl$). Found: *m*/*z*, 490.1166. $C_{21}H_{26}^{35}ClN_3O_3SNa$ requires *m*/*z*, 490.1179.

3.1.3. (S)-[2-[(5-nitro-1,3-thiazol-2-yl)carbamoyl]phenyl]-2-amino-3,3-dimethylbutanoate, hydrochloride RM-5061 ***(8a)***

The preceding Boc derivative **7a** (0.254 g, 0.53 mmol) was suspended in CH_2Cl_2 (5 mL) and 4 M HCl in dioxane (2 mL) was added with stirring at 20 °C. A solution resulted after a few minutes, but solid soon began to precipitate. After 16 h, the reaction was diluted with ether, briefly stirred, then cooled to 0 °C to complete precipitation; filtration afforded the title compound RM5061 **8a** (0.205 g, 93%); ^1H NMR [400 MHz, $(CD_3)SO$] δ_H 1.10 (9 H, s, Me_3C), 4.00 (1 H, br s, *CH*NH_3^+), 7.54 (1 H, d, J = 8.0 Hz, ArH), 7.62 (1 H, t, J = 8.0 Hz, ArH), 7.75 (1 H, t, J = 8.0 Hz, ArH), 7.85 (1 H, d, J = 8.0 Hz, ArH), 8.73 (1 H, s, thiazole 4-H), 8.86 (3 H, br s, NH_3^+) and 13.85 (1 H, br s, NH); ^{13}C NMR [100 MHz, $(CD_3)SO$] δ_C 26.6, 33.9, 61.5, 124.0, 126.6, 127.1, 130.0, 133.7, 142.6, 143.0, 147.8, 162.2, 165.8 and 167.5; *m*/*z* (ES + ve mode) 379 (base peak, ammonium ion). Found: C, 46.1; H, 4.6; N, 13.6. $C_{16}H_{19}N_4O_5SCl$ requires C, 46.3; H, 4.6; N, 13.5%; Found: *m*/*z*, 379.1060. $C_{16}H_{19}N_4O_5S$ requires *m*/*z*, 379.1076.

The highest ee's were observed when DMF was used in the coupling step. Following deprotection, RM5061 **8a** was obtained with an HPLC area purity of 99.0% and a chiral purity of 99.8%. The corresponding (R) enantiomer, viz. derived from D-*tert*-leucine, was similarly made; this material had an HPLC purity of 99.5% and a chiral purity of 99.5%.

3.1.4. (S)-[2-[(5-chloro-1,3-thiazol-2-yl)carbamoyl]phenyl]-2-amino-3,3-dimethylbutanoate, hydrochloride RM-5064 ***(8b)***

This compound was prepared similarly to **8a**. From **7b** (600 mg, 1.28 mmol) there was obtained HCl salt RM 5064 **8b** (490 mg, 94%); ^1H NMR [400 MHz, $(CD_3)SO$] δ_H 1.07 (9 H, s, Me_3C), 3.96 (1 H, d, J = 7.6 Hz, *CH*NH), 7.47 (1 H, t, J = 8.0 Hz, ArH), 7.56 (1 H, d, J = 8.0 Hz, ArH), 7.60 (1 H, s, 4′-H), 7.68 (1 H, t, J = 8.0 Hz, ArH), 7.75 (1 H, d, J = 8.0 Hz, ArH) and 8.82 (3 H, br s, NH_3^+); ^{13}C NMR [100 MHz, $(CD_3)SO$] δ_C 26.6, 33.8, 61.6, 119.1, 123.8, 127.0, 127.5, 129.7, 133.0, 136.2, 147.7, 156.3, 164.8 and 167.4; *m*/*z* (ES + ve mode) 368 (base peak, ammonium ion). Found: *m*/*z*, 368.0833. $C_{16}H_{19}N_3O_3S^{35}Cl$ requires *m*/*z*, 370.0806.

For the synthesis and characterization of compounds (**5a**), (**9**) and (**10**) and their Boc precursors, together with details of the pharmacokinetic and toxicological methods employed, see Supporting Information.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.09.080.

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