Eur. J. Med. Chem. 35 (2000) 487–497

S022352340000146X/FLA

Original article

Growth hormone secretagogues derived from NN703 with hydrazides as c-terminal

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Received 10 September 1999; revised 29 November 1999; accepted 1 December 1999

Abstract – A series of GH secretagogues based on modifications in the C-terminal of NN703 is reported. The C-terminal N-methyl amide of NN703 has been replaced with alkylated hydrazides in order to decrease the volume of distribution and identify GH secretagogues with shorter duration of action. Most of the prepared compounds show high potency in a rat pituitary assay. Subsequent to an initial in vivo screening in dogs, four compounds were selected for further pharmacological and pharmacokinetic evaluation. The four compounds showed oral bioavailability around 35% and equipotency in vitro compared to NN703. The relationship between lipophilicity and volume of distribution is discussed and it is speculated whether the lower volume of distribution is attributed to the observed higher in vivo potency and shorter plasma elimination half-life. © 2000 Éditions scientifiques et médicales Elsevier SAS

growth hormone releasing peptides / growth hormone secretagogue / NN703 / hydrazides / MK677 / GHRP / volume of distribution / half-life

1. Introduction

Since Momany and Bowers [1–3] discovered a series of peptides that specifically released growth hormone (GH) from the rat pituitary, a number of small peptides and non-peptides with similar effect have been found [4]. These compounds (generally identified as GHS or GHRP) have shown GH releasing capability in a number of species, including man [5]. It is well established that their mechanism of action differs from that of GHRH [6, 7]. Prominent members of the GH secretagogue family (*figure 1*) are the peptides GHRP-1, GHRP-2, GHRP-6 [3], hexarelin [8] and ipamorelin [9], the non-peptides L-692,429 (**1**) [10], MK677 (**2**) [11] and the acylated dipeptide, NN703 (**3**) [12]. The GH secretagogues were discovered via 'reverse pharmacology' [13] or more precisely 'reverse drug discovery', referring to the fact that a putative endogenous ligand corresponding to these synthetic compounds has not been reported yet, whereas a receptor (GHS 1A) with high affinity for the GH secretagogues has been identified and cloned recently [14, 15].

Compounds **2** and **3** are systematically available after oral administration (i.e. oral bioavailable) and this is clearly a point of utmost importance if a GH secretagogue shall enter the market in competition with the existing recombinant GH therapy [16]. Another issue of high importance is based on a previously proposed link between the plasma elimination half-life and receptor-desensitization and magnitude of GH-induced IGF-I release [17]. For instance, chronic dosing of **2** in humans results in down regulation of the GH release but still exhibits prolonged elevation of the IGF-I level [11]. This has been associated with the relatively long plasma elimination half-life of **2** ($t_{½}$ ~ 6 h). In this context it is noteworthy that short acting compounds such as GHRP-2 have demonstrated efficacy with regard to growth velocity in children despite the absence of a sustained IGF-I

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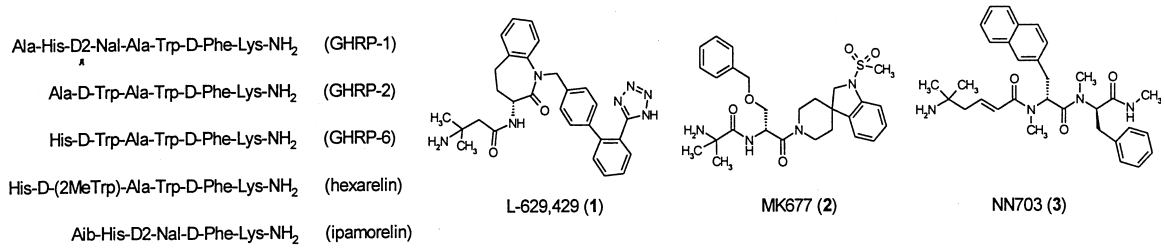


Figure 1. Some of the most prominent members of the GH secretagogue family.

elevation [18]. At present it is not clear exactly which pharmacological profile is optimal.

NN703 is a highly specific compound, has a long plasma elimination half-life ($t_{½}$ ~ 4 h) similar to that of **2** and a rather high volume of distribution (V_z ~ 8 L/kg) in beagles. Consequently, we see down-regulation of GH-release with NN703, as is the case with **2** [12]. This led us to search for compounds in the NN703 series with similar good in vitro and in vivo potencies, good oral bioavailability but a significantly shorter half-life. Since the half-life is related to the volume of distribution ($V_z = CL/\lambda_z$; $t_{½} = \ln 2/\lambda_z$), and the volume of distribution is believed to be related to the fat/water partition, we speculated whether a decrease in lipophilicity, provided that clearance is unchanged or decreased, would decrease such volume of distribution. Since NN703 is a rather lipophilic compound (clogP ~ 4.2) we hypothesised that a moderate increase in hydrophilicity could lead to the desired change in pharmacokinetic parameters. This was clearly a narrow balance as we had optimized the oral bioavailability of the NN703 series by applying 'the rule of five' [19], and we realized that major changes in size and polarity could easily be detrimental to the oral bioavailability. Therefore, we chose to replace the C-terminal amide with various hydrazides, because a hydrazide would maintain some of the amide character, which was important according to our SAR [20], and it would add an extra charge group to the molecule and supposedly increase polarity. This transformation was also synthetically easy and would not lead to a major increase in molecular weight. According to our SAR the C-terminal was also the region of NN703 that was most tolerant with regard to addition of extra steric bulk [20].

Those considerations led to the present series of analogues of **3** with small hydrazides in the C-terminal combined with isosteric modifications in the naphthyl and the phenyl moieties [21].

2. Chemistry

The GH secretagogues were prepared by stepwise peptide synthesis using Fmoc as the protecting group for N-methyl-D-2-phenylalanine (N-Me-D-Phe-OH), N-methyl-D-4-biphenylalanine (N-Me-D-Bip-OH), N-methyl-D-thienylalanine (N-Me-D-Thi-OH) and N-methyl-D-naphthylalanine (N-Me-D-2-Nal-OH) in the two first coupling steps (*figure 2*). Fmoc-N-Me-D-Phe-OH or Fmoc-N-Me-D-Thi-OH were readily coupled with various substituted hydrazines [22] using the standard EDC/HOBT coupling procedure. Removal of the FMOC protecting group with piperidine in dimethylformamide (DMF) resulted in the corresponding hydrazide in good yields. The resulting hydrazide was coupled to Fmoc-N-Me-D-2-Nal-OH or Fmoc-N-Me-D-Bip-OH using the same coupling procedure. Boc-protection was used in the final coupling step introducing 5-amino-5-methylhex-2-enoic acid. The final deprotection using trifluoroacetic acid in methylene chloride was carried out with care (as previously described) [20] affording the desired acylated dipeptides **4–20**.

3. Results and discussion

Due to the assumed influence of pharmacokinetic parameters on GH down-regulation and IGF-I prolongation [17], GH secretagogues derived from **3** with shorter duration of action were required. Therefore, the role of the C-terminal amide of **3** with respect to potency and pharmacokinetics was investigated by replacing the C-terminal amide with various hydrazides. The introduction of a basic moiety was hoped only to influence the in vitro and in vivo potency and oral bioavailability to a minor extent, but to have a major impact on the pharmacokinetic profile. The volume of distribution is related to clearance and plasma elimination half-life and is believed to be related to the lipophilicity of the compound. Often

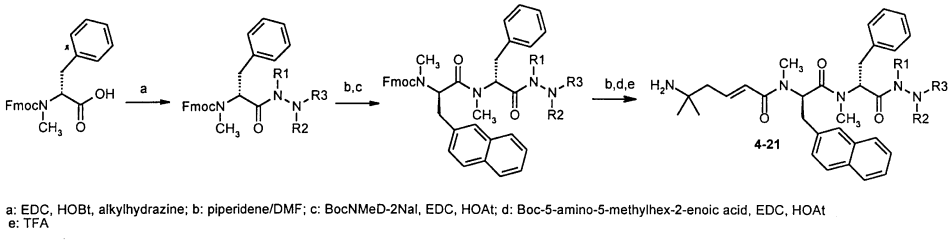


Figure 2. The first two coupling steps of the synthesis.

compounds which are relatively more polar would tend not to distribute to fatty tissues and exhibit a lower volume of distribution [23] and consequently to a shorter half-life, provided the clearance remain relatively unaffected. Thus, a series of various hydrazide analogues of **3** was prepared and tested in vitro and a selection of these was further pharmacologically and pharmacokinetically evaluated.

Growth hormone in vitro was measured in rat pituitary cells as described previously [9]. In this assay and in this series of experiments, the reference compound **3** had a potency (EC_{50}) of 8.3 ± 1.3 nM (mean ± SEM, n = 4) and an efficacy (E_{max}) of 100% of that measured for GHRP-6. In this assay GHRP-6 had a potency at 2.7 ± 0.8 nM; **1** was 169 ± 53 nM and **2** was 1.8 ± 0.5 nM.

Table I shows the in vitro potency (EC_{50}) and efficacy (E_{max}) of a series of hydrazide analogues of **3**. A number of derivatives are equipotent to **3**, such as **4**, **5**, **8**, **12**, **14** and **18** (EC_{50}s below 10 nM), while others are slightly less potent, such as **7**, **9**, **10**, **11**, **13** and **17** (EC_{50}s above 40 nM). With respect to efficacy, **4**, **10**, **11** and **17** are more efficacious in vitro than the other compounds (E_{max} > 100%).

The series contains simple methylated hydrazides (**5**, **8**, **14**, **16**, **18** and **20)**, hydrazides holding an additional carbonyl (**4**, **6**, **7** and **11**) and hydrazides holding bulky groups (**9**, **10**, **11**, **12**, **13**, **15**, **17** and **19**). In general, all compounds are full agonists (i.e. E_{max} > 80%), and despite the introduction of a basic moiety, an additional carbonyl or a bulky group in the C-terminal, no major changes in in vitro potency were observed. This may suggest the presence of a space between the C-terminal and the receptor, which is insensitive to the presence of such different groups as amines, carbonyls and lipophilic groups. Such additional free space may be used for further enhancement of the interaction of the compounds and the receptor.

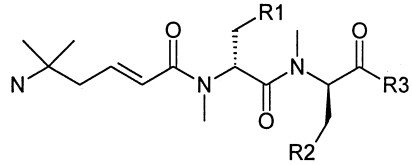
Since our main objective was to identify in vivo active GH secretagogues with good oral bioavailability and shorter half-life than **3**, we screened a selection of the compounds in dogs after oral administration.

3.1. In vivo pharmacology and pharmacokinetics in dogs

Our setup used fasted dogs that were dosed by gavage as well as by intravenous administration in a hind leg vein [12]. We have previously shown that at a dose of 2.5 mg/kg, compound **3** released 12.2 ± 9.9 ng/mL GH in dogs after oral administration (mean ± SEM, four dogs) [12]. To get a fast overview of the most interesting compounds, we screened several compounds (*table II*) in four dogs after oral administration of 1.0 mg/kg. Our selection criterion for further pharmacological and pharmacokinetic studies in this initial screening was a mean GH release above 10 ng/mL. As shown in *table II* the compounds **5**, **8**, **14**, **16**, **17**, **18** and **20** met this criterion. We selected **5**, **8**, **14** and **18** for further pharmacological and pharmacokinetic evaluation. These four compounds are structurally different to **3**, either they contain a trimethylhydrazine (e.g. **5** and **18**) or N,N-dimethylhydrazine (e.g. **8** and **14**) instead of methylamine, or that the phenylalanine has been replaced with a thienylalanine in combination with di- or trimethylhydrazine (e.g. **14** and **18**). The four compounds were tested in dogs after oral administration of a dose between 2.0 and 2.8 mg/kg and an i.v. administration at 0.5 mg/kg and compared with data for **3**. The results are shown in *table II*. All four compounds had slightly lower clearance (however, only **5** was significantly different to **3**) and while **8**, **14** and **18** had a significantly lower half-life, **5** showed a longer half-life, although not significantly different to **3**.

The volume of distribution (V_z) was derived from the half-life and the clearance (i.e. $V_z = CL/\lambda_z$; $t½ = \ln 2/\lambda_z$) and the V_z was lower for all four compounds, although only **18** was significantly lower than **3**. All four compounds had an oral bioavailability similar to **3**, and all four compounds showed a better GH release after oral administration than **3**, although none of them was significantly higher.

Table I. Potency (EC_{50}) and efficacy (E_{max}) of a series of GH secretagogues with modifications in the C terminal. Mean values of at least two separate experiments.



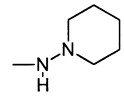
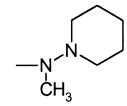
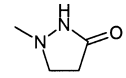
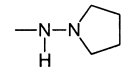
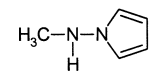
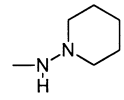
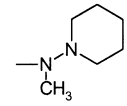
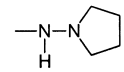
Entry	R1	R2	R3	EC_{50} (nM)	E_{max} (%)
3 (NN703)	2-naphthalene	phenylene	$-NHCH_3$	8	100
4	2-naphthalene	phenylene	$-NHNHCOCH_3$	8	125
5 (NNC 26-1089)	2-naphthalene	phenylene	$-NCH_3N(CH3)_2$	9	85
6	2-naphthalene	phenylene	$-NHNCH_3COCH_3$	17	90
7	2-naphthalene	phenylene	$-NCH_3NHCOCH_3$	28	95
8 (NNC 26-1136)	2-naphthalene	phenylene	$-NHN(CH_3)_2$	7	90
9	2-naphthalene	phenylene		24	90
10	2-naphthalene	phenylene		24	110
11	2-naphthalene	phenylene		22	105
12	2-naphthalene	phenylene		9	80
13	2-naphthalene	phenylene		35	85
14 (NNC 26-1137)	2-naphthalene	2-thienyl	$-NHN(CH_3)_2$	8	95
15	4-biphenylene	phenylene		11	100
16	4-biphenylene	phenylene	$-NCH_3N(CH_3)_2$	10	95
17	2-naphthalene	2-thienyl		24	110
18 (NNC 26-1167)	2-naphthalene	2-thienyl	$-NCH_3N(CH_3)_2$	9	85
19	4-biphenylene	phenylene		15	100
20	4-biphenylene	2-thienyl	$-NCH_3N(CH_3)_2$	13	80

Table II. GH release (ng/mL) after the initial screening in dogs (n = 4) after oral administration of 1.0 mg/kg of **4–20**, oral bioavailability (Fpo), volume of distribution (V_z), half-life ($t_{½}$), clearance (CL) and GH release after oral administration of 2.0–2.8 mg/kg of **3**, **5, 8, 14** and **18**, respectively. Values are mean ± SEM.

Entry	Screening (1.0 mg/kg)	Fpo (%)	$t_{½}$ (h)	CL (l/h/kg)	V_z (l/kg)	GH release (C_{max}) (ng/mL)
3 (NN703)		33 ± 6	4.1 ± 0.4	1.4 ± 0.2	8.2 ± 1.6	12.2 ± 5.0 (2.5 mg/kg)
4	< 10					
5 (NNC 26-1089)	> 10	32 ± 10	6.6 ± 1.5	0.6 ± 0.1*	5.6 ± 0.7	18.5 ± 4.9 (2.5 mg/kg)
6	> 10					
7	< 10					
8 (NNC 26-1136)	> 10	35 ± 3	2.7 ± 0.3*	0.8 ± 0.3	3.5 ± 1.5	21.1 ± 6.8 (2.0 mg/kg)
10	< 10					
11	< 10					
12	< 10					
14 (NNC 26-1137)	> 10	28 ± 4	2.5 ± 0.2*	0.8 ± 0.3	3.0 ± 1.3*	23.2 ± 9.3 (2.0 mg/kg)
15	< 10					
16	> 10					
17	> 10					
18 (NNC 26-1167)	> 10	33 ± 5	1.9 ± 0.2*	0.8 ± 0.2	2.26 ± 0.7*	21.0 ± 0.9 (2.8 mg/kg)
19	< 10					
20	> 10					

* $P > 0.05$ using student's t-test.

In total, all four compounds showed lower volume of distribution. The lower V_z was in all cases associated with lower clearance and shorter half-life, except for **5**, which surprisingly showed a longer half-life, but this was counteracted by a significantly lower clearance. The generally lower V_z is, however, in accord with our hypothesis that the introduction of a basic moiety into **3** would give a significantly lower volume of distribution and that this decrease would be reflected by a shorter half-life, given that the clearance is unchanged or decreased.

Meanwhile, one would expect **8** and **14** to be less lipophilic than **5** and **18** since these two compounds have an additional proton donor at the hydrazide, and according to the hypothesis this should lead to a lower V_z. This is only the case for **8** compared to **5** and is not the case for **14** compared to **18**, although no significant differences are observed. However, if the two trimethylhydrazides **5** and **18** are compared with respect to V_z, the introduction of a thienyl group seems to significantly decrease the V_z, whereas in the two dimethylhydrazides **8** compared to **14** no significant difference was observed. And compared to **3**, only the thienyl containing compounds (**14** and **18**) are significantly different, suggesting that the lower V_z may be attributed to the thienyl group rather than the hydrazides.

With respect to clearance, only **5** was significantly different to **3**, while no significant difference was observed between **8**, **14** and **18** to **3** and no significant difference was observed between the four hydrazides.

Meanwhile, our main purpose of the study was to identify compounds with a shorter half-life, and as shown in *table II*, the compounds **8**, **14** and **18** all showed significantly shorter half-lives than **3**, while **5** was not significantly different to **3** but significantly higher than the other hydrazides.

Overall, this could indicate that the substitution of a proton donor (as in the secondary amide in **3**) with a positive charge alone (i.e. **5**) is not enough to obtain a shorter half-life, but that the combination of a positive charge with a proton donor (i.e. **8** and **14**) or combined with a thienyl group (i.e. **18**) in our series is necessary.

The obvious reference compound in this study would be the thienyl analogue of NN703. This compound has previously been prepared and studied in a different set-up, but the compound did not meet the criterion for further biological evaluation (due to low GH release after oral administration) and therefore no data for comparison is accessible [20].

The in vivo potency of all four hydrazides was increased, although not significantly, and it may be speculated whether this is a reflection of the lower volume of distribution as well. It is likely that in this case, where the clearance is only slightly affected, the lower volume of distribution is directly translated into a higher amount of substance in systemic circulation and this subsequently results in a higher in vivo potency. Also it is interesting to note that despite the introduction of an additional positive charge, the oral bioavailability was unchanged. The in vitro specificity of **5** and **18** were evaluated in more than

100 receptor and enzyme assays and no significant binding at 10 μM was observed.

4. Conclusion

We have described a series of GH secretagogues derived from **3** (NN703) with hydrazides in the C-terminal. Based on the GH release from a rat pituitary assay and some initial in vivo screening, we selected the hydrazide analogues **5**, **8**, **14** and **18** for further in vivo characterization. In beagle dogs, all four compounds showed improved potency after oral administration of 2.0–2.8 mg/kg, with respect to GH release and similar oral bioavailability compared to **3**. Meanwhile, the volume of distribution of all four compounds and the half-life of all but **5** was significantly lower than **3**, and we speculate whether the decreased volume of distribution may explain the relatively higher in vivo potency. Therefore, we have succeeded in our main goal in identifying new GH secretagogues derived from NN703 with improved in vivo potency and shorter plasma elimination half-lives.

5. Experimental procedure

The four amino acids have been purchased from Synthetech Inc. (PO Box 646, Albany, Oregon 97321) or prepared as previously described [20]. All hydrazines used were commercially available, except trimethylhydrazine and 1-(methylamino)piperidine, which were prepared by formylation of unsym-dimethylhydrazine and 1-aminopiperidine, respectively, followed by reduction with lithiumaluminumhydride according to a procedure by Class et al. [22].

High performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR, Bruker 400 MHz) or liquid chromatography-mass spectrometry (LC-MS) confirms the structures of the compounds. NMR shifts (δ) are given in parts per million (ppm) and only selected peaks are given. M.p. is melting point and is given in °C. The methanol/ammonia solution used is a 10% ammonia solution in methanol.

5.1. HPLC methods

HPLC (method A1): the RP-HPLC analysis was performed using UV detection at 214 nm and a Hibar LiChrosorb RP-18 (5 uM) 250-4 (Merck) column, which was eluted at 1 mL/min. Two solvent systems were used: solvent system I: 0.1% trifluoroacetic acid in acetonitrile. Solvent system II: 0.1% trifluoroacetic acid in water. The column was equilibrated with a mixture composed of 5% of solvent system I and 95% of solvent system II. After injection of the sample a gradient of 20–80% of solvent system I in solvent system II was run over 30 min. The gradient was then extended to 100% of solvent system I over 5 min followed by isocratic elution with 100% of this system for 5 min.

The RP-analysis was performed using UV detection at 214, 254, 276 and 301 nm on a 218TP54 4.6 × 250 mm 5 μ C-18 silica column (The Seperations Group, Hesperia), which was eluted at 1 mL/min at 42 °C. The column was equilibrated with 5% acetonitrile in a buffer consisting of 0.1 M ammonium sulfate, which was adjusted to pH 2.5 with 4 M sulfuric acid. After injection the sample was eluted by a gradient of 5–60% acetonitrile in the same buffer for 50 min.

HPLC (method B1): the RP-HPLC analysis was performed using UV detection at 214 nm and a Hibar LiChrosorb RP-18 (5uM) 250-4 (Merck) column, which was eluted at 1 mL/min. Two solvent systems were used: solvent system I: 0.1% trifluoroacetic acid in acetonitrile. Solvent system II: 0.1% trifluoroacetic acid in water. The column was equilibrated with a mixture composed of 5% solvent system I and 95% solvent system II. After injection of the sample a gradient of 20–80% of solvent system I in solvent system II was run over 30 min. The gradient was then extended to 100% of solvent system I over 5 min followed by isocratic elution with 100% of this system for 5 min.

The RP-analysis was performed using UV detection at 214, 254, 276 and 301 nm on a 218TP54 4.6 × 250 mm 5 μ C-18 silica column (The Seperations Group, Hesperia), which was eluted at 1 mL/min at 42 °C. The column was equilibrated with 5% (acetonitrile + 0.1% TFA) in an aqueous solution of TFA in water (0.1%). After injection the sample was eluted by a gradient of 5–60% (acetonitrile + 0.1% TFA) in the same aqueous buffer for 50 min.

HPLC (method H8): the HPLC analyses was performed using a Waters® millenium system using a Water® 3 × 150 mm 3.5 μ C-18 Symmetry column. The column was heated to 42 °C and eluted with a linear gradient of 5–90% acetonitrile, 85–0% water and 10% trifluoroacetic acid (0.5%) in water in 15 min at a flow-rate of 1 mL/min.

The LC-MS analyses were performed on a PE Sciex API 100 LC/MS System using a Waters® 3 × 150 mm 3.5 μ C-18 Symmetry column and positive ionspray with a flow rate of 20 μL/min. The column was eluted with a linear gradient of 5–90% acetonitrile, 85–0% water and 10% trifluoroacetic acid (0.1%)/water in 15 min at a flow rate of 1 mL/min.

*5.2. (2E)-5-Amino-5-methylhex-2-enoic acid N-((1R)-1-(N-[(1R)-2-(N′-acetylhydrazino)-1-benzyl-2-oxoethyl]-N-methylcarbamoyl)-2-(2-naphthyl)ethyl)-N-methylamide (**4**)*

To a solution of *tert*-butyl carbazate (1.0 g, 7.6 mmol) and pyridine (3.1 mL) in methylene chloride (5 mL) was slowly added acetic acid anhydride (1.5 mL) and the mixture was stirred overnight. The mixture was added to methylene chloride (50 mL) and washed with water (2 × 10 mL) and brine (10 mL) and dried ($MgSO_4$), filtered and concentrated in vacuo to give 0.95 g of N′-acetylhydrazinecarboxylic acid *tert*-butyl ester as a yellow oil. LC-MS: R_t = 5.39, m/z = 349.6 (m + 1); ^{1}H-NMR ($CDCl_3$) selected peaks: δ 1.5 (s, 9H, (CH_3)$_3$C–O); 2.05 (s, 3H, CH_3CO).

N′-Acetylhydrazinecarboxylic acid *tert*-butyl ester (0.95 g, 5.45 mmol) was dissolved in methylene chloride (10 mL) and trifluoroacetic acid (10 mL) was added and the mixture was stirred at room temperature for 1 h. The mixture was concentrated in vavuo and stripped three times with methylene chloride to give 1.0 g of acetic acid hydrazide. Then (2R)-2-(*tert*-butoxycarbonylmethylamino)-3-phenyl propionic acid (0.76 g, 2.73 mmol) was dissolved in a mixture of dimethyl formamide (3 mL) and methylene chloride (6 mL) and a mixture of 1-hydroxy-7-azabenzotriazole (0.45 g, 3.28 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.62 g, 3.28 mmol) was added and stirred for 20 min. Then a mixture of acetic acid hydrazide (1.0 g, 5.45 mmol) and diisopropylethylamine (1.87 mL) was added and the mixture was stirred overnight. Ethyl acetate (50 mL) was added and the mixture was washed with water (50 mL). The aqueous layer was extracted with ethyl acetate (3 × 50 mL) and the combined organic layers were washed with water (2 × 50 mL) and brine (50 mL) and dried ($MgSO_4$), filtered, concentrated to an oil, and chromatographed on silica (40 g) with heptane:ethyl acetate (1:1) to give 0.81 g of *N*-[(1R)-2-(*N*′-acetylhydrazino)-1-benzyl-2-oxoethyl]-*N*-methylcarbamic acid *tert*-butyl ester as a yellow oil. LC-MS R_t = 9.34, m/z = 336.4 (m + 1); HPLC: R_t = 10.17 min (H8); ^{1}H-NMR ($CDCl_3$) selected peaks: δ 1.32 + 1.40 (2 s, 9H, (CH_3)$_3$C–O, rotamere); 2.05 (s, 3H, COCH_3); 2.78 (s, 3H, N-CH_3).

N-[(1R)-2-(*N*′-Acetylhydrazino)-1-benzyl-2-oxoethyl]-*N*-methylcarbamic acid *tert*-butyl ester (0.81 g, 2.42 mmol) was dissolved in methylene chloride (5 mL) and trifluoroacetic acid (5 mL) was added and the mixture was stirred for 30 min at room temperature. The mixture was concentrated in vacuo and stripped three times with methylene chloride and the remaining oil was chromatographed on silica (40 g) with methylene chloride: methanol/ammonia (9:1) to give 0.41 g of acetic acid N′-((2R)-2-(methylamino)-3-phenylpropionyl)hydrazide as an amorphous powder. Then (2R)-2-(*N*-*tert*-butoxycarbonyl-*N*-methylamino)-3-(2-naphthyl)propionic acid (0.63 g, 1.92 mmol) was dissolved in methylene chloride (10 mL) and a mixture of 1-hydroxy-7-azabenzotriazole (0.26 g, 1.92 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.37 g, 1.92 mmol) was added and stirred for 30 min. A mixture of acetic acid N′-((2R)-2-(methylamino)-3-phenylpropionyl)hydrazide (0.41 g, 1.74 mmol) and DIEA (0.39 mL) was added and the mixture was stirred overnight. Methylene chloride (50 mL) was added and the mixture was washed with water (50 mL). The organic layer was washed with aqueous sodium bicarbonate (10 mL) and brine (50 mL) and dried ($MgSO_4$), filtered, concentrated to an oil, and chromatographed on silica (40 g) with heptane:ethyl acetate (1:4) to give 0.59 g of *N*-((1R)-1-(*N*-[(1R)-2-(*N*′-acetylhydrazino)-1-benzyl-2-oxoethyl]-*N*-methylcarbamoyl)-2-(2-naphthyl)ethyl)-*N*-methylcarbamic acid *tert*-butyl ester as an oil. LC-MS R_t = 13.68 min, m/z = 547.2 (m + 1); HPLC: R_t = 13.53 min (H8).

To a solution of *N*-((1R)-1-(N-[(1R)-2-(*N*′-acetylhydrazino)-1-benzyl-2-oxoethyl]-*N*-methylcarbamoyl)-2-(2-naphthyl)ethyl)-*N*-methylcarbamic acid *tert*-butyl ester (0.59 g, 1.08 mmol) in methylene chloride (5 mL) was added trifluoroacetic acid (5 mL) at 0 °C and stirred for 90 min. The mixture was concentrated in vacuo and stripped three times with methylene chloride. The obtained oil was dissolved in methanol/ammonia (2 mL) and added methylene chloride (20 mL) and silica gel (5 g) and concentrated in vacuo. The obtained powder was extracted by filtration with methylene chloride (100 mL) and methylene chloride:methanol/ammonia (9:1) and the combined extracts were concentrated in vacuo to 0.41 g of crude product as a foam. Then (2E)-5-(*tert*-butyloxycarbonylamino)-5-methylhex-2-enoic acid (0.21 g, 0.89 mmol) was dissolved in methylene chloride (10 mL) and a mixture of 1-hydroxy-7-azabenzotriazole (0.13 g, 0.98 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (0.17 g, 0.89 mmol) was added and stirred for 30 min. A mixture of the above crude product (0.40 g, 0.89 mmol) and diisopropylethylamine (0.20 mL) was added and the mixture was stirred overnight. Methylene chloride (50 mL) was added and the mixture was washed with water (10 mL). The organic layer was washed with aqueous sodium bicarbonate (10 mL) and brine (50 mL), dried ($MgSO_4$), filtered, concentrated to 0.42 g of a tan foam. The foam was dissolved in methylene chloride (5 mL), cooled to 0 °C and trifluoroacetic acid (5 mL)

was added and the mixture was stirred for 45 min. The reaction mixture was quenched with methylene chloride (50 mL) and water (10 mL) and titrated to pH ~ 7 with solid sodium bicarbonate. The organic layer was separated and washed with water (10 mL), dried ($MgSO_4$), filtered and concentrated in vacuo. The obtained product was dissolved in acetonitrile/water 1:20 (10 mL) and applied to a C-18 Sep-Pak Classic© cartridge (2.0 g, purchased from Waters™), which had been prewashed with acetonitrile (100 mL) and water (100 mL). Then a gradient of an eluent consisting of water/acetonitrile/trifluoroacetic acid (10, 15, 20 and 25% acetonitrile in water/0.1% trifluoroacetic acid) was run through the Sep-Pak© (2 g). The relevant fractions were combined and lyophilised to 0.19 g of the trifluoroacetic acid salt of (2E)-5-amino-5-methylhex-2-enoic acid *N*-((1R)-1-(*N*-[(1R)-2-(*N*′-acetylhydrazino)-1-benzyl-2-oxoethyl]-*N*-methylcarbamoyl)-2-(2-naphthyl)ethyl)-*N*-methylamide as a white amorphous powder. LC-MS: R_t = 9.21 min, m/z = 572.4 (m + 1); HPLC: R_t = 26.45 min (A1), R_t = 28.40 (B1).

*5.3. (2E)-5-Amino-5-methylhex-2-enoic acid N-methyl-N-((1R)-1-(N-methyl-N-[(1R)-2-phenyl-1-(N,N′,N′′-trimethylhydrazinocarbonyl)ethyl]-carbamoyl)-2-(2-naphthyl)ethyl)amide (***5***)*

To a solution of *tert*-butyl carbazate (1.0 g, 7.56 mmol) in anhydrous tetrahydrofuran (40 mL) at 0 °C was added sodium hydride (60% dispersion in oil, 2.73 g, 68 mmol) and methyl iodide (11.3 mL, 181 mmol) and the mixture was stirred for 3 days. Tetrahydrofuran (100 mL) was added and the suspension was filtrated and the filtrate was concentrated in vacuo. The obtained product was dissolved in ethyl acetate and chromatographed on silica gel (40 g) with heptane:ethyl acetate (1:1) and concentrated to give 0.53 g (40%) of N,N′,N′-trimethylhydrazinecarboxylic acid *tert*-butyl ester as a thin oil. ^{1}H-NMR ($CDCl_3$): δ 1.48 (s, 9H, $(CH_3)_3C$–O) 2.6 (s, 6H, 2 N–CH_3) 2.9 (s, 3H, N–CH_3).

To a solution of N,N′,N′-trimethylhydrazinecarboxylic acid *tert*-butyl ester (0.52 g, 2.99 mmol) in methylene chloride (4 mL) was added trifluoroacetic acid (4 mL) and the mixture was stirred for 60 min. The mixture was concentrated in vacuo and stripped three times with methylene chloride to give 0.61 g of *N*,*N*,*N*′-trimethylhydrazine trifluoroacetate as a thin oil. Then (2R)-2-(*tert*-butoxycarbonylmethylamino)-3-phenyl propionic acid (1.0 g, 3.58 mmol) was dissolved in methylene chloride (5 mL) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.34 g, 1.79 mmol) was added and stirred for 30 min. Then a mixture of *N*,*N*,*N*′-trimethylhydrazine trifluoro acetate (0.37 g, 1.96 mmol) and diisopropylethylamine (0.92 mL, 5.34 mmol) was added and the mixture was stirred for 2 days. Methylene chloride (50 mL) was added and the mixture was washed with water (50 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (2 × 10 mL) and water (50 mL), dried ($MgSO_4$), filtered, concentrated in vacuo, and chromatographed on silica (100 g) with heptane:ethyl acetate (1:1) to give 0.30 g *N*-methyl-*N*-[(1R)-2-phenyl-1-(*N*,*N*′,*N*′-trimethylhydrazinocarbonyl)ethyl]carbamic acid *tert*-butyl ester as an amorphous powder. HPLC: R_t = 13.03 min (H8); ^{1}H-NMR ($CDCl_3$) selected peaks: δ 1.23 + 1.35 (2 s, 9H, $(CH_3)_3C$–O, rotamere) 2.25 + 2.42 + 2.45 (3 s, 6H, N-N($CH_3)_2$, rotamere).

To a solution of *N*-methyl-*N*-[(1R)-2-phenyl-1-(*N*,*N*′,*N*′-trimethylhydrazinocarbonyl)ethyl]carbamic acid *tert*-butyl ester (0.3 g, 0.89 mmol) in methylene chloride (1 mL) was added trifluoroacetic acid (1 mL) and the mixture was stirred for 30 min. The mixture was concentrated in vacuo, stripped three times with methylene chloride and suspended in methylene chloride:methanol/ammonia (1:1) (2 mL) and trifluoroammonium acetate precipitated. Then diethyl ether (10 mL) was added and the mixture was filtered and the filtrate was concentrated in vacuo to give 0.31 g of (2R)-2-methylamino-3-phenylpropionic acid trimethylhydrazide as an oil. Then (2R)-2-(*N*-*tert*-butoxycarbonyl-*N*-methylamino)-3-(2-naphthyl)propionic acid (0.35 g, 1.07 mmol) was dissolved in methylene chloride (20 mL) and a mixture of 1-hydroxy-7-azabenzotriazole (0.15 g, 1.07 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.21 g, 1.07 mmol) was added and stirred for 30 min. Then a mixture of (2R)-2-methylamino-3-phenylpropionic acid trimethylhydrazide (0.21 g, 0.89 mmol) and diisopropylethylamine (0.20 mL) was added and the mixture was stirred overnight. Methylene chloride (30 mL) was added and the mixture was washed with water (10 mL). The organic layer was washed with aqueous sodium bicarbonate (10 mL) and brine (50 mL), dried ($MgSO_4$), filtered, concentrated to an oil, and chromatographed on silica (40 g) with heptane:ethyl acetate (3:7) to give 0.43 g of *N*-methyl-*N*-((1R)-1-(*N*-methyl-*N*-[(1R)-2-phenyl-1-(*N*,*N*′,*N*′-trimethylhydrazinocarbonyl)ethyl]carbamoyl)-2-(2-naphthyl)ethyl)carbamic acid *tert*-butyl ester as an oil.^{1}H-NMR ($CDCl_3$) selected peaks: δ 0.92 + 1.22 (2 s, 9H, $(CH_3)_3C$–O, rotamere) 5.0 + 5.3 (2 t, 1H, C*H*-$CH_2C_6H_5$, rotamere) 6.3 + 6.45 (2 t, 1H, C*H*-$CH_2C_{10}H_7$, rotamere).

To a solution of *N*-methyl-*N*-((1R)-1-(*N*-methyl-*N*-[(1R)-2-phenyl-1-(*N*,*N*′,*N*′-trimethylhydrazinocarbonyl)ethyl]carbamoyl)-2-(2-naphthyl)ethyl)carbamic acid *tert*-

butyl ester (0.43 g, 0.79 mmol) in methylene chloride (2 mL) was added trifluoroacetic acid (2 mL) at 0 °C and stirred for 30 min. The mixture was concentrated in vacuo and stripped three times with methylene chloride. The obtained oil was dissolved in methanol/ammonia (2 mL) and added methylene chloride (20 mL) and silica gel (5 g) and concentrated in vacuo. The obtained powder was extracted by filtration with methylene chloride (50 mL) and methylene chloride:methanol/ammonia (9:1) and the combined extracts were concentrated in vacuo to 0.36 g of crude product as a foam. Then (2E)-5-(*tert*-butyloxycarbonylamino)-5-methylhex-2-enoic acid (0.12 g, 0.48 mmol) was dissolved in methylene chloride (5 mL) and a mixture of 1-hydroxy-7-azabenzotriazole (0.065 g, 0.48 mmol) and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (0.092 g, 0.48 mmol) was added and stirred for 30 min. Then a mixture of the obtained crude product (0.18 g, 0.40 mmol) and diisopropylethylamine (0.09 mL, 0.53 mmol) was added and the mixture was stirred overnight. Methylene chloride (50 mL) was added and the mixture was washed with water (10 mL). The organic layer was washed with aqueous sodium bicarbonate (10 mL) and brine (50 mL), dried ($MgSO_4$), filtered, concentrated to 0.14 g of a colourless oil. The oil was dissolved in methylene chloride (2 mL), cooled to 0 °C and trifluoroacetic acid (2 mL) was added and the mixture was stirred for 30 min. The reaction mixture was quenched with methylene chloride (50 mL) and water (10 mL) and titrated to pH ~ 7 with solid sodium bicarbonate. The organic layer was separated and washed with water (10 mL), dried ($MgSO_4$), filtered and concentrated in vacuo. The obtained product was dissolved in acetonitrile/water 1:20 (10 mL) and applied to a C-18 Sep-Pak Classic© cartridge (2.0 g, purchased from Waters™), which had been prewashed with acetonitrile (100 mL) and water (100 mL). Then a gradient of an eluent consisting of water/acetonitrile/trifluoroacetic acid (10, 15, 20 and 25% acetonitrile in water/trifluoroacetic acid) was run through the Sep-Pak©. The relevant fractions were combined and lyophilised to 0.072 g of the trifluoroacetic acid salt of (2E)-5-amino-5-methylhex-2-enoic acid *N*-methyl-*N*-((1R)-1-(*N*-methyl-*N*-[(1R)-2-phenyl-1-(*N*,*N*′,*N*′-trimethylhydrazinocarbonyl)ethyl]carbamoyl)-2-(2-naphthyl)ethyl)amide as a white amorphous powder. LC-MS: R_t = 9.96 min, m/z = 572.4 (m + 1). HPLC: R_t = 35.23 min (A1), R_t = 37.45 (B1); ^{1}H-NMR (DMSO) selected peaks: δ 5.1 (t, 1H, C*H*-$CH_2C_6H_5$) 6.1 (t, 1H, CH_2–C*H*=C) 6.2 (d, 1H, C=C*H*–CO) 6.4 (t, 1H, C*H*–$CH_2C_{10}H_7$); anal ($C_{34}H_{45}N_5O_3$ x 2 $C_2H_4O_2$ x ½ H_2O) Calc: C 65.12; H 7.77; N 9.99; Found: C 64.54; H7.73; N 9.97.

The compounds listed below were all prepared by the general procedure above. The purity (determined by HPLC) was generally in the 95–100% range.

*5.4. (2E)-5-Amino-5-methylhex-2-enoic acid N-((1R)-1-(N-[(1R)-2-(N′-acetyl-N′-methylhydrazino)-1-benzyl-2-oxoethyl]-N-methylcarbamoyl)-2-(2-naphthyl)ethyl)-N-methylamide (***6***)*

LC-MS: R_t = 9.255 min, m/z = 586.4 (m + 1); HPLC: R_t = 31.47 min (A1), R_t = 33.30 (B1); ^{1}H-NMR (DMSO) selected peaks: 5.3 (dd, 1H, C=C*H*–CO) 5.7 (t, 1H, C*H*–$CH_2C_6H_5$) 6.15 (d, 1H, CH_2–C*H*=C) 6.4 (m, 1H, C*H*–$CH_2C_{10}H_7$).

*5.5. (2E)-5-Amino-5-methylhex-2-enoic acid N-((1R)-1-(N-[(1R)-2-(N′-acetyl-N-methylhydrazino)-1-benzyl-2-oxoethyl]-N-methylcarbamoyl)-2-(2-naphthyl)ethyl)-N-methylamide (***7***)*

LC-MS: R_t = 8.74 min, m/z = 586.4 (m + 1); HPLC: R_t = 29.53 (A1); R_t = 31.35 (B1); ^{1}H-NMR (DMSO) selected peaks: δ 1.05 (s, 6H, C–(CH_3)$_2$); 1.77 (s, 3H, COCH_3); 6.2 (d, 1H, C=C*H*–CO); 6.3 (m, 1H, CH_2–C*H*=C).

*5.6. (2E)-5-Amino-5-methylhex-2-enoic acid N-((1R)-1-(N-[(1R)-1-(N′,N′-dimethylhydrazino-carbonyl)-2-phenylethyl]-N-methylcarbamoyl)-2-(2-naphthyl)ethyl)-N-methylamide (***8***)*

HPLC: R_t = 30.42 min (A1), R_t = 30.38 (B1), R_t = 8.40 min (H8); LC-MS: R_t = 8.99 min, m/z = 558.

*5.7. (2E)-5-Amino-5-methylhex-2-enoic acid N-methyl-N-((1R)-1-{N-methyl-N-[(1R)-2-phenyl-1-((piperidin-1-yl)carbamoyl)ethyl]carbamoyl}-2-(2-naphthyl)ethyl)amide (***9***)*

HPLC: Rt = 34.78 min (A1), Rt = 33.74 min (B1); LC-MS: Rt = 9.81 min, m/z = 598.4 (m + 1).

*5.8. (2E)-5-Amino-5-methylhex-2-enoic acid N-methyl-N-((1R)-1-(N-methyl-N-[(1R)-1-(N-methyl-N-(piperidin-1-yl)carbamoyl)-2-phenylethyl]-carbamoyl)-2-(2-naphthyl)ethyl)amide (***10***)*

HPLC: Rt = 38.85 min (A1), Rt = 40.19 min (B1), Rt = 11.42 min (H8); LC-MS: Rt = 12.11 min, m/z = 612.4 (m + 1).

*5.9. (2E)-5-Amino-5-methylhex-2-enoic acid N-((1R)-1-(N-[(1R)-1-benzyl-2-oxo-2-(3-oxopyrazolidin-1-yl)ethyl]-N-methylcarbamoyl)-2-(2-naphthyl)ethyl)-N-methylamide (**11**)*

HPLC: Rt = 29.21 min (A1), Rt = 8.43 min (H8); LC-MS: Rt = 9.24 min, m/z = 584.4 (m + 1).

*5.10. (2E)-5-Amino-5-methylhex-2-enoic acid N-methyl-N-((1R)-1-{N-methyl-N-[(1R)-2-phenyl-1-((pyrrolidin-1-yl)carbamoyl)ethyl]carbamoyl}-2-(2-naphthyl)ethyl)amide (**12**)*

HPLC: Rt = 34.80 min (A1), Rt = 34.04 min (B1); LC-MS: Rt = 9.46 min, m/z = 610.4 (m + 1).

*5.11. (2E)-5-Amino-5-methylhex-2-enoic acid N-methyl-N-((1R)-1-{N-methyl-N-[(1R)-2-phenyl-1-((pyrrol-1-yl)carbamoyl)ethyl]carbamoyl}-2-(2-naphthyl)ethyl)amide (**13**)*

HPLC: Rt = 35.94 min (A1), Rt = 37.55 min (B1); LC-MS: Rt = 10.14 min, m/z = 580.4 (m + 1).

*5.12. (2E)-5-Amino-5-methylhex-2-enoic acid N-((1R)-1-(N-[(1R)-1-(N′,N′-dimethylhydrazinocarbonyl)-2-(2-thienyl)ethyl]-N-methylcarbamoyl)-2-(2-naphthyl)ethyl)-N-methylamide (**14**)*

HPLC: Rt = 30.03 min (A1), Rt = 29.88 min (B1); LC-MS: Rt = 8.97 min, m/z = 564.4 (m + 1).

*5.13. (2E)-5-Amino-5-methylhex-2-enoic acid N-((1R)-2-(biphenyl-4-yl)-1-(N-methyl-N-[(1R)-2-phenyl-1-((piperidin-1-yl)carbamoyl)ethyl]-carbamoyl)ethyl)-N-methylamide (**15**)*

HPLC: Rt = 37.03 min (A1), Rt = 35.93 min (B1), Rt = 10.45 min (H8); LC-MS: Rt = 10.24 min, m/z = 624.4 (m + 1).

*5.14. (2E)-5-Amino-5-methylhex-2-enoic acid N-((1R)-2-(biphenyl-4-yl)-1-(N-methyl-N-[(1R)-2-phenyl-1-(N,N′,N′-trimethylhydrazinocarbonyl)-ethyl]carbamoyl)ethyl)-N-methylamide (**16**)*

HPLC: Rt = 37.23 min (A1), Rt = 38.88 min (B1), Rt = 10.88 min (H8); LC-MS: Rt = 11.14 min, m/z = 598.4 (m + 1).

*5.15. (2E)-5-Amino-5-methylhex-2-enoic acid N-methyl-N-((1R)-1-(N-methyl-N-[(1R)-1-(N-methyl-N-(piperidin-1-yl)carbamoyl)-2-(2-thienyl)ethyl]-carbamoyl)-2-(2-naphthyl)ethyl)amide (**17**)*

HPLC: Rt = 39.47 min (A1), Rt = 41.43 min (B1), Rt = 11.30 min (H8); LC-MS: Rt = 12.17 min, m/z = 618.4 (m + 1).

*5.16. (2E)-5-Amino-5-methylhex-2-enoic acid N-methyl-N-((1R)-1-(N-methyl-N-[(1R)-2-(2-thienyl)-1-(N,N′,N′-trimethylhydrazinocarbonyl)-ethyl]carbamoyl)-2-(2-naphthyl)ethyl)amide (**18**)*

HPLC: Rt = 33.85 min (A1), Rt = 35.38 min (B1); LC-MS: Rt = 10.02 min, m/z = 578.2 (m + 1).

*5.17. (2E)-5-Amino-5-methylhex-2-enoic acid N-((1R)-2-(biphenyl-4-yl)-1-{N-methyl-N-[(1R)-2-phenyl-1-((pyrrolidin-1-yl)carbamoyl)-ethyl]carbamoyl}ethyl)-N-methylamide (**19**)*

HPLC: Rt = 34.80 min (A1), Rt = 34.04 min (B1); LC-MS: Rt = 9.46 min, m/z = 610.4 (m + 1).

*5.18. (2E)-5-Amino-5-methylhex-2-enoic acid N-((1R)-2-(biphenyl-4-yl)-1-{N-methyl-N-[(1R)-2-(2-thienyl)-1-(N,N′,N′-trimethylhydrazinocarbonyl) ethyl]carbamoyl}ethyl)-N-methylamide (**20**)*

HPLC: Rt = 36.92 min (A1), Rt = 39.91 min (B1); LC-MS: Rt = 5.47 min, m/z = 604.2 (m + 1).

5.19. Pharmacokinetic and pharmacodynamic studies in dogs

Oral bioavailability studies were conducted in male and female beagle dogs. The dogs were fasted overnight prior to dosing. Diet was withheld for at least 3 h post dosing. A 1 week wash-out period separated p.o. and i.v. dosing. The compounds were administered in a vehicle of citrate/phosphate buffer, pH 5.0 by stomach tubing. For i.v. administration the dogs received a dose of 0.5 mg compound/kg as a bolus in a hind leg vein. EDTA blood samples were drawn from a front leg vein at intervals up to 23 h after dosing. Blood samples were placed on ice-water bath immediately after sampling. Plasma was separated by centrifugation and stored frozen pending analysis for canine GH and the compounds.

The oral bioavailability (f) was calculated as the total area under the plasma concentration versus time curve following p.o. administration divided by the area following i.v. administration, appropriately corrected for dose.

The AUC (0-tn) is the area under the plasma concentration curve from time zero to the last measurable concentration estimated by the trapezoidal rule. λ_z is the terminal elimination rate constant, calculated by log–linear regression. The systemic clearance, CL, is:

$$CL = dose_{iv}/AUC_{iv}$$

and the volume of distribution during the terminal phase, V_z, is:

$$V_z = dose_{iv}/(AUC_{iv} \cdot \lambda_z)$$

Acknowledgements

We would like to thank Peter Andersen (synthesis) Anette Heerwagen (in vitro pharmacology) and Edward Kristensen (in vivo pharmacology and pharmacokinetics) for excellent technical assistance.

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