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The discovery of biaryl carboxamides as novel small molecule agonists of the motilin receptor

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ABSTRACT

Optimisation of urea (**5**), identified from high throughput screening and subsequent array chemistry, has resulted in the identification of pyridine carboxamide (**33**) which is a potent motilin receptor agonist possessing favourable physicochemical and ADME profiles. Compound (**33**) has demonstrated prokinetic-like activity both in vitro and in vivo in the rabbit and therefore represents a promising novel small molecule motilin receptor agonist for further evaluation as a gastroprokinetic agent.

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Motilin is a 22-amino acid peptide secreted by the enterochromaffin cells of the small intestine and is the endogenous ligand of the 7-TM motilin receptor, previously known as GPR38.[1] This receptor is located primarily in the gastrointestinal tract on the enteric nerves, smooth muscle and gastric vagal nerve terminals with the highest levels present in the stomach and duodenum.[1–3] Motilin is thought to act as a 'house-keeper', promoting migrating motor complexes which sweep the GI tract in the fasted state.[4] However, it has also been shown that motilin[5] and other motilin receptor agonists[6] promote gastric emptying and propulsion of GI tract contents in an anal direction in the fed state. Consequently, motilin receptor agonists have the potential for the treatment of conditions where gastric and upper intestinal motility is impaired; for example, in certain subsets of patients suffering from gastroparesis[7] and functional dyspepsia[8] and also in patients in a critical care setting where treatment of GI stasis can result in improved recovery times.[9]

The antibiotic erythromycin (**1**) (Fig. 1) is also a motilin receptor agonist[10] and is frequently used in a clinical setting for the treatment of gastroparesis.[7] However, due to its antibiotic activity and potential for cardiac side-effects, it is not suitable for chronic use. Several groups have made extensive progress in modifying erythromycin giving a class of motilin receptor agonists known as the motilides.[11] Despite demonstrating improved gastric emptying in healthy volunteers, many of these motilides (e.g. KC11458,[12] ABT-229[13]) have suffered difficulties in the clinic. These have variously been ascribed to tachyphylaxis, dose selection/dosing regimen, inappropriate patient selection or potential selectivity issues.[11,14] However, two members of the motilide class, mitemcinal[15] (GM-611) (**2**) (Phase II) and PF-04548043/KOS-2197[14c] (Phase I) are currently progressing in clinical trials. Other groups have disclosed non-motilide agonists (**3, 4**)[16,17] but these are generally high molecular weight compounds (>550) (Fig. 1).

At GlaxoSmithKline, we embarked on a programme towards the discovery of small molecule motilin receptor agonists. Thus, a high throughput screening campaign and efficient array chemistry led to the discovery of (**5**)[18] (Fig. 2) which possessed good activity at the recombinant human motilin receptor (pEC_{50} 7.3)[19,20] and also promoted nerve-mediated contractile response in isolated rabbit gastric antrum. However, (**5**) also possessed high *c*log*P* and molecular weight and consequently, P450 inhibition[21] (3A4 IC_{50} 0.2 and 0.4 μM) and microsomal clearance profiles (CLi: human >50, rat 23 mL min^{-1} g^{-1} liver) were undesirable. In this letter we report

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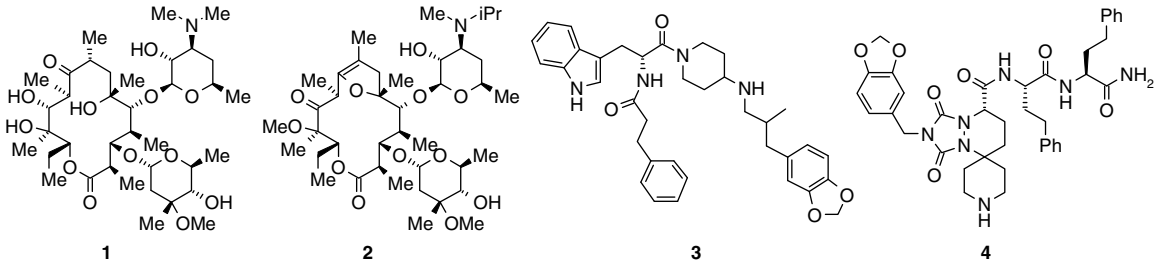


Figure 1. Erythromycin, mitemcinal and reported non-motilide motilin receptor agonists.

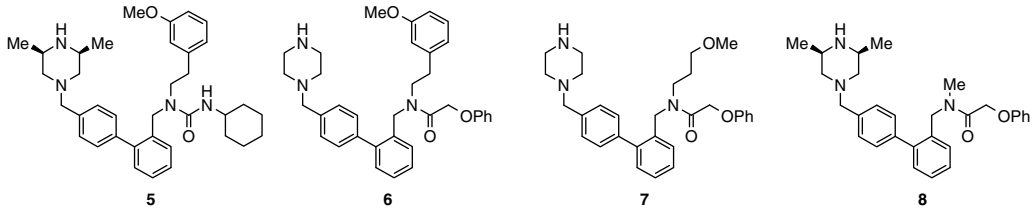


Figure 2. (Piperazinylmethyl)biphenylmethyl urea and amide motilin receptor agonists.

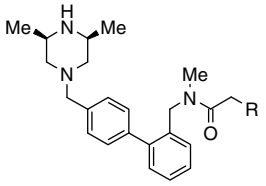
on further optimisation of urea (**5**) leading to the identification of pyridine- and imidazole-2-carboxamide agonists of the motilin receptor.

Preliminary SAR showed that the *cis*-2,6-dimethylpiperazine gave enhanced agonist potency at the motilin receptor when compared to an unsubstituted piperazine head group. We discovered that the presence of this group allowed us to replace one of the side-chains in the moderately active amides (**6**) and (**7**)[18] (Fig. 2) with a simple methyl group to give compound (**8**) which possessed reduced molecular weight and ~3- to 5-fold improvement in potency when compared with (**6**) and (**7**). In comparison, replacement of the 3-methoxyphenethyl side-chain of (**5**) with a methyl group resulted in a 40-fold drop in agonist potency (data not shown). Therefore, a variety of other amide analogues of (**8**) were prepared (Table 1).

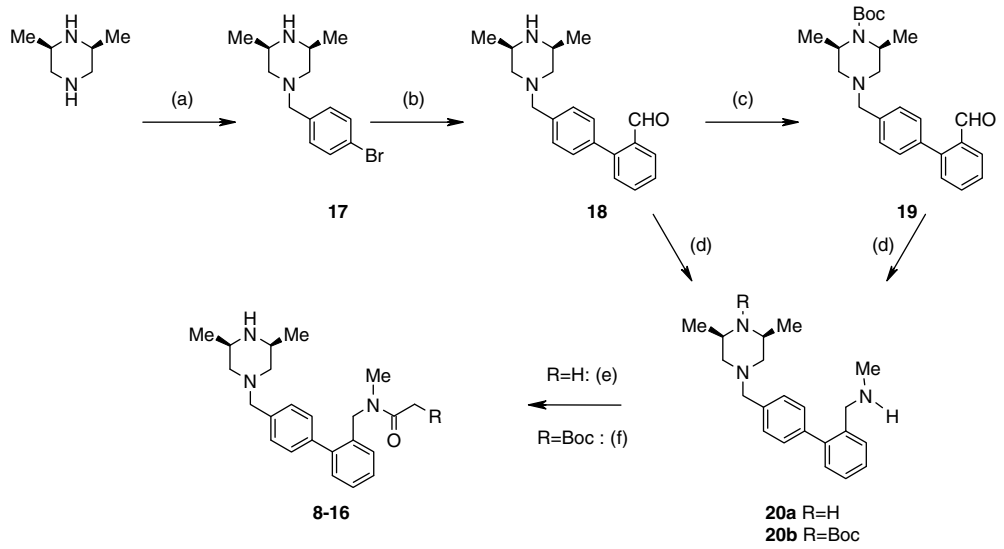
The 4-fluoro-analogue (**9**) showed a 2.5-fold improvement in potency compared to (**8**) but removal of the oxygen linker from the side-chain of (**9**) to give (**10**) resulted in a slight drop in potency. A variety of other linkers as exemplified by (**12**)–(**14**) also gave good levels of agonist activity but the importance of the optionally substituted phenyl group at the terminus of the side-chain was demonstrated by compound (**15**) where R is *iso*butyl such that a drop in potency was observed despite the compound having a *c*log*P* similar to that of compound (**8**). Introduction of a higher degree of polarity into the terminal ring was also detrimental to potency, for example see isoxazole (**16**).

We were pleased to observe that reduction in *c*log*P* also led to an improvement in the in vitro ADME profiles for some of these analogues, Table 1. For those compounds possessing the terminal phenyl ring in the side-chain, phenoxyacetamide (**8**) showed much improved P450 3A4 inhibition and microsomal clearance profiles when compared to urea (**5**). Replacement of the oxygen linker in (**8**) with a sulfone (**13**) gave increased potency and a further drop in *c*log*P* although this was not accompanied by a significant

Table 1
Agonist activity (FLIPR) at human motilin receptor,[20] microsomal clearance and P450 inhibition profiles[21] of amide derivatives (**8**)–(**16**).[22]

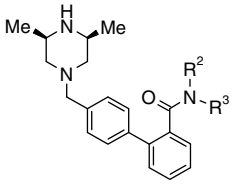


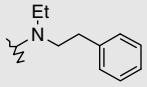
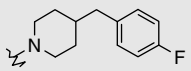
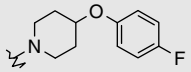
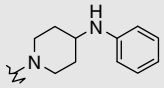
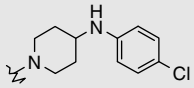
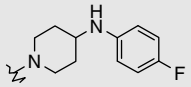
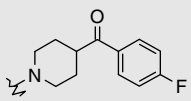
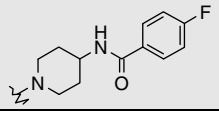
Compound	R	M_W	*c*log*P*	hMotilinR pEC$_{50}$	CLi (mL min^{-1} g^{-1}) human, rat	P450 3A4 IC$_{50}$ (μM)
5	–	568	7.4	7.3	>50, 23	0.2, 0.4
8	PhO–	457	5.3	7.1	4.9, 9.5	7.0, 24
9	4-F-PhO–	475	5.6	7.5	–	6.0, 7.3
10	4-F-Ph–	459	5.2	7.3	–	–
11	3-Cl-Ph–	476	5.7	7.1	20, 17	1.4, 1.5
12	PhS–	473	5.8	8.5	–	–
13	PhSO$_2$–	506	4.3	8.0	6.8, 4.3	3, 8.9
14	(4-MePh)SO$_2$NH–	534	5.0	7.7	–	–
15	^{i}PrCH$_2$–	421	5.3	6.6	–	–
16	3-Me-5-isoxazoyl–	446	3.3	6.0	2.4, 2.9	40, >100



Scheme 1. Reagents and conditions: (a) 4-bromobenzaldehyde, $NaBH(OAc)_3$, DCM, 0 °C–rt, 87%; (b) 2-formylbenzene boronic acid, 3 M Na_2CO_{3aq}, $Pd(PPh_3)_4$, 1,2-DME, 80 °C, 90%; (c) Boc anhydride, Et_3N, DCM, rt, 68%; (d) $MeNH_2$ (8 M in THF), MeOH, rt, 16 h then $NaBH_4$, rt, 82% R = H, 69% R = Boc; (e) RCH_2CO_2H, PS-carbodiimide, HOBt, DCM, rt, 2–13%; (f) RCH_2CO_2H, PS-carbodiimide, HOBt, DCM, rt then HCl/Et_2O, DCM, rt, 25–78%.

Table 2
Agonist activity (FLIPR) at human motilin receptor,[20] microsomal clearance and P450 inhibition profiles[21] of amide derivatives (**21**)–(**28**).[22]

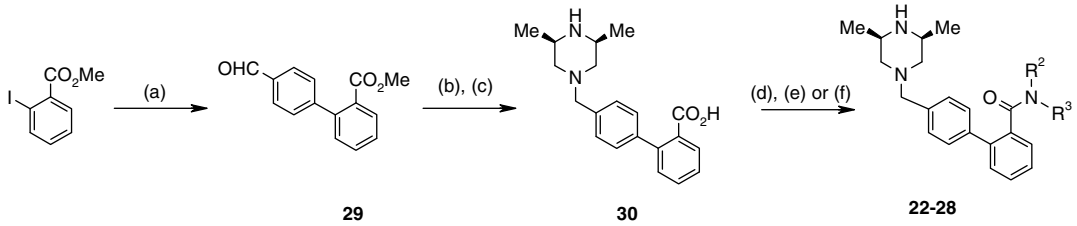


Compound	$-NR^2R^3$	*c*log*P*	hMotilinR pEC_{50}	CLi (mL min^{-1} g^{-1}) human, rat	P450 3A4 IC_{50} (μM)
21		5.9	8.1	40, 26	0.9, 1.5
22		6.5	8.0	29, 7	0.6, 0.5
23		5.5	7.2	—	8.4, 14
24		4.8	8.3	7, 20	12, 17
25		5.8	7.3	8, 33	5.2, 3.5
26		5.2	8.6	—	—
27		4.9	7.2	—	4.8, 3.6
28		4.3	<5	—	—

improvement in the in vitro ADME profile. Replacement of the terminal phenyl ring with a more polar heteroaromatic such as the isoxazole (**16**) resulted in a favourable ADME profile with low microsomal clearance and P450 3A4 inhibition. However, since the potency of this compound was much reduced, we were faced with finding a delicate balance between the degree and position of lipophilicity required for motilin agonist activity and the nature and position of more polar functionality which would give acceptable in vitro ADME properties.

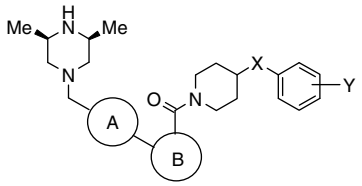
The synthesis of amide analogues (**8**)–(**16**) was undertaken as shown in Scheme 1. Reductive amination of 4-bromobenzaldehyde with *cis*-2,6-dimethylpiperazine followed by standard Suzuki–Miyaura coupling with 2-formylbenzeneboronic acid gave aldehyde (**18**). Further reductive amination with methylamine gave key intermediate (**20a**) which was converted to products (**8**), (**10**) and (**16**) by coupling with the appropriate acids in the presence of polymer-supported carbodiimide and hydroxybenzotriazole. Due to the low yields obtained for the final step in the synthesis of these three analogues, a modified route was used for the remaining analogues. The piperazine in (**18**) was Boc-protected before reductive amination with methylamine to give (**20b**). Amide coupling and final Boc-deprotection then occurred in improved overall yields to give compounds (**9**) and (**11**)–(**15**).

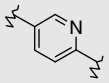
Compounds (**8**)–(**16**) possessed two benzylamino- groups which was potentially sub-optimal from a metabolic stability perspective. We had previously shown that the LHS benzylpiperazine moiety was a requirement for motilin agonist activity.[18] Therefore, we proceeded to investigate replacement of the benzylic amide on the RHS of the molecule through preparation of the reversed amides. Compound (**21**),[23] Table 2, showed improved agonist potency at the motilin receptor compared with analogues (**10**) and



Scheme 2. Reagents and conditions: (a) 4-formylbenzeneboronic acid, Na_2CO_3, $Pd(OAc)_2$, nBu_4NBr, 1,2-DME/H_2O (1:1), microwave, 150 °C, 10 min, 75%; (b) *cis*-2,6-dimethylpiperazine, $NaBH(OAc)_3$, DCM, rt, 98%; (c) 2M $NaOH_{aq}$, EtOH, rt, 77%; (d) compounds (**22**), (**25**) and (**28**): R^1R^2NH, EDCI.HCl, HOBt, DMF, 50 °C, 25–42%; (e) compounds (**23**), (**24**) and (**27**): R^1R^2NH, PS-carbodiimide, HOBt, DMF, 50 °C, 6–36%; (f) compound (**26**): R^1R^2NH, EDCI.HCl, DMAP, DCM/DMF, rt, 19%.

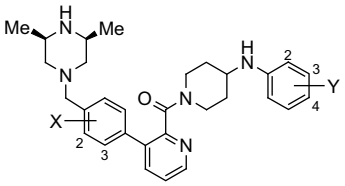
Table 3
Agonist activity (FLIPR) at human motilin receptor,[20] microsomal clearance and P450 inhibition profiles[21] of amide derivatives (**31**)–(**36**).[22]



Compound	A[a]	B[a]	X	Y	*c*log*P*	hMotilinR pEC_{50}	CLi (mL min^{-1} g^{-1}) human, rat	P450 3A4 IC_{50} (μM)
31		Ph	NH	4-F	4.0	<5	–	–
32		Ph	NH	4-F	3.8	6.4	–	–
33	Ph		NH	4-F	4.1	7.7	2.6, 2.8	>100, >100
34	Ph		NH	3-F	3.6	7.8	3.4, 4.1	58, 46
35	Ph		NH	3-F	4.3	5.6	–	–
36	Ph		CH_2	4-F	5.4	8.0	16, 16	13, 5.6

[a] When A is phenyl the regiochemistry is 1,4. When B is phenyl the regiochemistry is 1,2.

Table 4
Agonist activity (FLIPR) at human motilin receptor[20] and microsomal clearance data for amide derivatives (**37**)–(**47**).[22]



Compound	X	Y	*c*log*P*	hMotilinR pEC_{50}	CLi (h, r) (mL min^{-1} g^{-1})
33	H	4-F	4.1	7.7	2.6, 2.8
37	H	3-F	4.1	8.2	4.4, 6.5
38	H	2-F	4.1	7.3	—
39	H	4-CN	3.8	6.2	—
40	H	3-CN	3.8	7.7	3.5, 4.6
41	H	2-CN	3.8	6.1	—
42	H	4-$CONH_2$	2.5	5.6	—
43	H	3,4-diF	4.3	8.7	7.0, 5.9
44	H	4-OMe	3.8	6.0	—
45	2-F	4-F	4.3	8.4	5.5, 6.5
46	2-F	3-F	4.3	8.0	9.4, 12.0
47	2-OMe	4-F	4.1	8.3	5.3, 5.9

h, human; r, rat.

Table 5
Further profiling of amides (**33**) and (**34**).

Property	**33**	**34**
P450 IC_{50} (μM)	1A2, 2C9, 2C19 >100; 2D6 15	1A2 74; 2C9, 2C19 >100; 2D6 29
hERG binding pIC_{50}	<4.5	<4.6
hGhrelinR pEC_{50}	7.0	6.3
M_W	501	490
Log*D* (pH 7.4)	0.72	0.82
Aq solubility (HCl salt) (mg/mL)	⩾1	⩾0.5

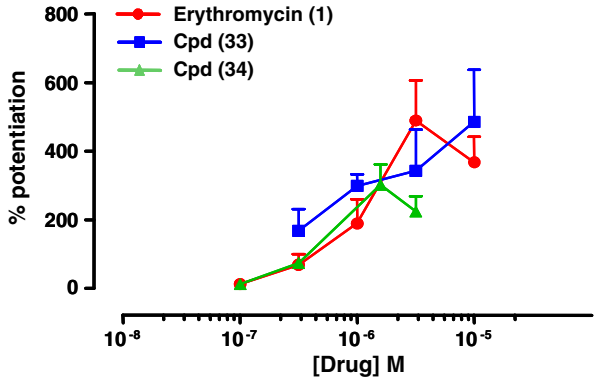


Figure 3. Prokinetic-like activity of erythromycin (**1**), compounds (**33**) and (**34**) on isolated rabbit gastric antrum.

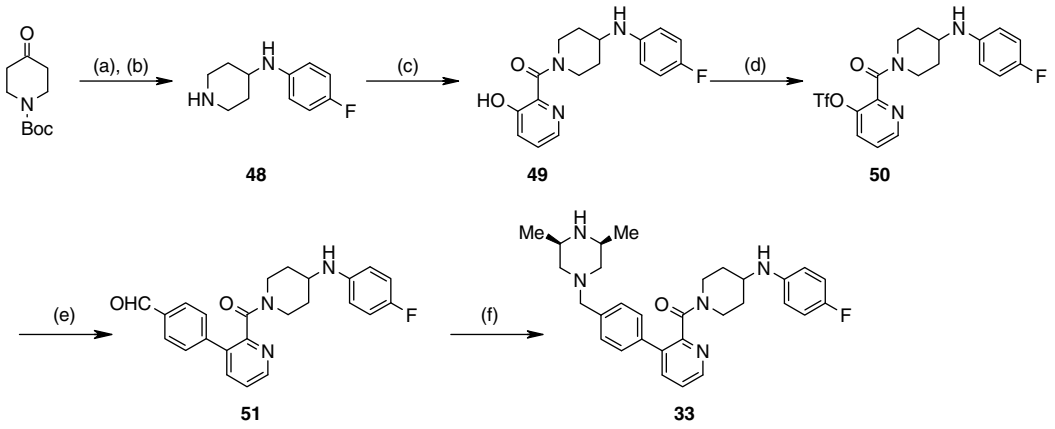
Table 6
Prokinetic-like activity of erythromycin (**1**), compounds (**33**) and (**34**) on isolated rabbit gastric antrum.

Compound	Concentration (μM)	Potentiation (%)	*N*[a]
1	10	368 ± 74	5
	3	490 ± 117	5
	1	189 ± 71	5
	0.3	69 ± 32	5
33	10	486 ± 151	3
	3	343 ± 120	3
	1	299 ± 33	3
	0.3	168 ± 63	3
34	3	224 ± 45	4
	1.5	302 ± 60	4
	0.3	73 ± 12	3

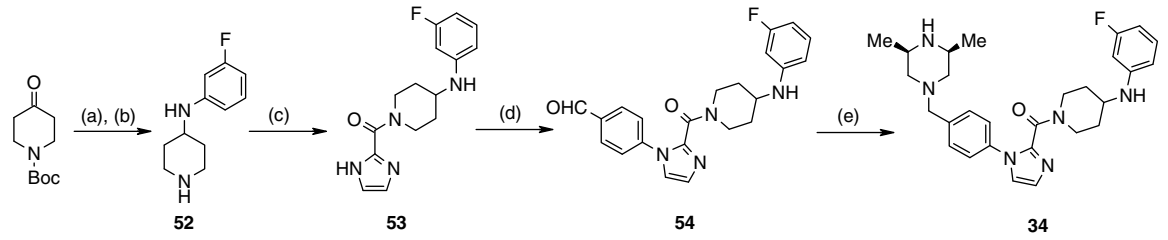
[a] *N*, number of strips of gastric antrum tissue used per test conc.

(**11**). However, removal of the RHS benzylic centre offered no improvement in microsomal clearance levels despite (**21**) possessing a similar overall level of lipophilicity to (**11**).

Conformational constraint of the RHS of this series of reversed amides was also examined through preparation of 4-substituted piperidine amide derivatives (**22**)–(**28**) as shown in Scheme 2. The methylene linked compound (**22**) showed an overall profile similar to that of non-constrained analogue (**21**) with excellent potency at the motilin receptor (pEC_{50} 8.0), high *c*log*P*, high microsomal clearance and significant P450 3A4 inhibition, Table 2. However, it was apparent that the nature of the linking atom between the piperidine and the terminal phenyl ring had a marked effect on both potency and in vitro ADME properties. Replacement of the benzylic terminal group with a phenoxy group (**23**) gave a 10-fold reduction in overall lipophilicity together with a 6-fold reduction in potency but we were pleased to observe that P450 3A4 inhibition was also reduced by >10-fold. A further reduction in overall lipophilicity was achieved by replacement of the ether linker with an amino linker as in (**24**). This compound showed improved agonist potency and a similar P450 3A4 inhibition profile to (**23**) but microsomal clearance was still too high. We were somewhat surprised to observe that the 4-chloro analogue (**25**) was 10-fold less potent than the unsubstituted analogue (**24**) despite being 10-fold more lipophilic and that the microsomal clearance was not significantly affected by the presence of this substituent. In the limited set of compounds prepared in this series, 4-fluoro substitution (**26**) gave the best potency with pEC_{50} 8.6 and this



Scheme 3. Reagents and conditions: (a) (i) 4-fluoroaniline, AcOH, 1,2-DCE, rt, (ii) $NaBH(OAc)_3$, 1,2-DCE, rt, 96%; (b) 2 M HCl, 1,4-dioxane, 60 °C, quant; (c) (i) 3-hydroxypyridine-2-carboxylic acid, $^{i}BuOCOCl$, Et_3N, DCM, (ii) (**48**), iii—2 M $NaOH_{aq}$, 94%; (d) *N*-phenyl *bis*(trifluoromethylsulfonimide), Et_3N, DCM, quant; (e) 4-formylbenzene boronic acid, Na_2CO_3, $Pd(PPh_3)_4$, 1,2-DME, H_2O, 50 °C, quant.; (f)*cis*-2,6-dimethylpiperazine, $NaBH(OAc)_3$, 1,2-DCE, rt, 63%.



Scheme 4. Reagents and conditions: (a) (i) 3-fluoroaniline, AcOH, 1,2-DCE, rt, (ii) $NaBH(OAc)_3$, 1,2-DCE, rt, 96%; (b) 2 M HCl, 1,4-dioxane, 60 °C, quant; (c) ethyl imidazole-2-carboxylate, Me_3Al, PhMe, rt, 54%; (d) 4-formylbenzene boronic acid $[Cu(OH).TMEDA]_2Cl_2$, O_2, 1,2-DCE, 50 °C, 87%; (e)*cis*-2,6-dimethylpiperazine, AcOH, 1,2-DCE, 60 °C, then $NaBH(OAc)_3$, 1,2-DCE, rt, 52%.

group together with the 3-fluoro analogue were used in further investigations.

Modification of the (phenylamino)piperidine amide series targeting further improvement of the in vitro ADME profile was carried out by replacing each of the core phenyl rings with a selection of heteroaromatic or heterocyclic rings as exemplified in Table 3. Replacement of the phenyl 'A-ring' with 3-pyridyl gave an inactive compound (**31**); however, the thiazole analogue (**32**) was better tolerated although potency was still lower than we required. Attention was then turned to analogues where the 'B-ring' phenyl was modified. Replacement of the phenyl 'B-ring' with pyridine (**33**) resulted in a 10-fold drop in lipophilicity and we were pleased to observe that this change was not only tolerated with respect to agonist potency at the motilin receptor (pEC_{50} 7.7), but also gave a much improved P450 3A4 inhibition and microsomal clearance profiles. The analogous imidazole (**34**) showed a similar level of potency but the pyrrolidine analogue (**35**) was considerably less potent despite possessing a similar overall level of lipophilicity. The beneficial effect of combining the amino linker with the pyridyl 'B-ring' is also illustrated by comparison of (**33**) with the benzyl analogue (**36**). The 20-fold increase in lipophilicity gave only a marginal increase in potency but was significantly detrimental to the in vitro ADME profile.

The SAR around pyridyl compound (**33**) was further explored as shown in Table 4. It was found that various alternative substitution patterns resulted in higher potency at the motilin receptor, for example compounds (**37**), (**43**) and (**45**)–(**47**). The *c*log*P* values for these compounds were either the same as, or not significantly higher than for (**33**), but the addition of extra substituents in the 2-position of the phenyl 'A-ring' or the terminal phenyl ring was detrimental for in vitro metabolic stability. Therefore, (**33**) and the imidazole (**34**) were selected for further profiling as shown in Table 5. In addition, these compounds showed >100-fold selectivity against a variety of receptor, ion channel and enzyme targets except for the closely related ghrelin receptor[24] where (**33**) showed 5-fold selectivity (pEC_{50} 7.0) and (**34**) showed 30-fold selectivity (pEC_{50} 6.3).

The in vivo pharmacokinetic properties of (**33**) and (**34**) were assessed. In the rat, pyridine (**33**) was a moderate clearance compound (CL_b 53 mL min^{-1} kg^{-1}) with 13% oral bioavailability (F_{po}). However, on dosing to the dog a much improved profile was observed, particularly with regard to oral absorption; (**33**) still showed moderate clearance (CL_b 19 mL min^{-1} kg^{-1}) but its oral bioavailability was 58% in this species. Imidazole (**34**) showed a similar profile to (**33**) in the rat with CL_b 61 mL min^{-1} kg^{-1} and F_{po} 17%.

The gastric prokinetic-like activity of (**33**) and (**34**) was also assessed whereby the compounds were tested for their effects on contractions evoked by electrical-field-stimulation (EFS) of isolated rabbit gastric antrum tissue.[25] This type of stimulation results in nerve-mediated contractions of the muscle and both motilin and erythromycin have demonstrated a robust ability to facilitate the amplitude of contractions in this assay. Thus, application of a test compound results in potentiation of the contractions because of its ability to activate the motilin receptors located on cholinergic neurons in this tissue. Figure 3 and Table 6 show the effects of (**33**) and (**34**), compared to erythromycin, when applied to the tissue at a range of concentrations.

These data show that in the range of 0.3–10 μM, pyridine (**33**) possessed a profile (potentiation 486 ± 151% at 10 uM) comparable to that of erythromycin (E_{max} 490 ± 117% at 3 μM). At a higher concentration of 30 μM, compound (**33**) showed a still greater potentiation with E_{max} 1260 ± 60% (n = 4) (data not shown in graph for clarity). Whilst the imidazole (**34**) was somewhat less efficacious,

its maximal effect (E_{max} 302 ± 60% at 1.5 μM) was still similar to the effect of (**33**) at a similar concentration. Although (**33**) showed a sub-optimal level of selectivity at the ghrelin receptor, it has previously been demonstrated that ghrelin does not show any contractile activity on rabbit gastric antrum under EFS conditions.[26] Hence, the prokinetic-like activity observed with (**33**) in this assay may be attributed to its motilin receptor agonist activity.

Compounds (**33**) and (**34**) were prepared according to Schemes 3 and 4, respectively.[27] The RHS 4-arylaminopiperidines were synthesised via standard reductive amination and deprotection methods. For compound (**33**), piperidine (**48**) was coupled with 3-hydroxypicolinic acid using isobutyl chloroformate to activate the acid. A basic hydrolysis was also required after the coupling to hydrolyse the isobutyl ester formed in the initial step back to the free hydroxy compound (**49**). Activation of the hydroxyl as the triflate (**50**) was followed by standard Suzuki–Miyaura coupling to give the biaryl core (**51**) in quantitative yield. Finally, a further reductive amination step with *cis*-2,6-dimethylpiperazine proceeded regioselectively to give compound (**33**). The imidazole (**34**) was prepared using a similar strategy whereby the piperidine (**52**) was coupled with ethyl imidazole-2-carboxylate in the presence of trimethylaluminium to give (**53**) in good yield. Reaction of imidazole (**53**) with 4-formylbenzeneboronic acid utilising a copper-mediated oxidative coupling[28] resulted in the formation of the biaryl core (**54**) in excellent yield. As for compound (**33**), a final reductive amination step furnished the target compound (**34**).

In summary, starting from urea lead (**5**) a strategy involving modulation of lipophilicity in such a way as to maintain activity at the motilin receptor resulted in the identification of amides possessing a key heteroaryl-containing core and 4-(phenylamino)piperidine RHS. In particular, compounds (**33**) and (**34**) combined good agonist activity at the motilin receptor with favourable in vitro DMPK and physicochemical profiles. Pyridyl analogue (**33**) also showed a highly promising level of prokinetic-like activity in isolated rabbit gastric antrum and this, in combination with its in vivo pharmacokinetic profile, has resulted in further assessment of the compound in an in vivo rabbit model of whole gut transit. In this model, (**33**) showed a statistically significant increase in both the number and weight of faecal pellets produced by the rabbits at 2 h after a 3 mg/kg iv bolus dose of the compound.[29]

In conclusion, compound (**33**), GSK326416,[30] represents an exciting novel small molecule motilin receptor agonist for further evaluation as a gastric prokinetic agent.

References and notes

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19. Potency data quoted in this paper was obtained using a FLIPR assay format with recombinant human motilin receptor stably expressed in a HEK293 cell line[20] whereas data quoted in the previous publication (Ref. 18) used a CHO cell line. pEC_{50} values represent the mean from at least three independent experiments with SEM ⩽ 0.2 (SD ⩽ 0.3) in all cases except compound (**25**) where SEM = 0.2 (SD = 0.4, n = 5) and compounds (**8**), (**10**), (**24**), (**31**), (**35**), (**44**) and (**47**), where n = 2. Comparison of activities of standard compounds in these assay formats:

Cell line	Human motilin pEC_{50} (n)	Erythromycin pEC_{50} (n)	Compound **5** pEC_{50} (n)
HEK293	9.3 (77)	6.2 (7)	7.3 (17)
CHO	10.4 (770)	7.3 (4)	8.0 (80)

20. FLIPR assay protocol: HEK-293 cells stably expressing the human motilin receptor were seeded (30,000 cells/100 μL growth media/well) into poly-D-lysine coated 96-well black-wall, clear-bottom microtitre plates (Corning) 24 h prior to assay. On the day of assay the cells were loaded with 2 μM (final) Fluo-4-AM fluorescent indicator dye (Molecular Probes) and 1 mM (final) probenicid in assay buffer (145 mM sodium chloride, 2.5 mM potassium chloride, 10 mM Hepes, 10 mM glucose, 1.2 mM magnesium chloride, 1.5 mM calcium chloride and 0.1% BSA) (50 μL loading solution added to each well). Plates were incubated for 1 h at 25 °C, before being washed four times with 100 μL assay buffer using the EMBLA cell washer; 150 μL residual being left after the final wash. The cells were then incubated at 25 °C for 20 min and the plates were then assayed on a Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices). Test compounds were prepared in assay buffer without probenecid. In the FLIPR, 50 μL of test compound was added to the cells and changes in fluorescence measured over a 2-min timeframe. Maximum change in fluorescence over baseline was used to determine agonist response and concentration response curves were constructed, using a 4-parameter logistic equation. All compounds were full agonists in this assay.
21. P450 data determined using Gentest enzyme source/assay format. Two IC_{50} values are quoted for 3A4 corresponding to inhibition of turnover of two substrates, DEF (Diethoxyfluorescein) and PPR (Phenylpiperazinylmethylbenzylresofurin), respectively.
22. All new compounds gave satisfactory ^{1}H NMR and mass spectral data.
23. Compound (**21**) was prepared in five steps as follows: (i) phenethylamine, acetyl chloride, triethylamine, DCM, rt, quant; (ii) $LiAlH_4$, THF, 50 °C, 98%; (iii) 2-IC_6H_4 COCl, DIPEA, DCM, rt, 76%; (iv) 4-$(OHC)C_6H_4B(OH)_2$, $Pd(PPh_3)_4$, 3 M Na_2CO_{3aq}, DME, reflux, 46%; (v) *cis*-2,6-dimethylpiperazine, DCM, rt, 1 h then $NaBH(OAc)_3$, rt, 72%.
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30. Characterising data for (**33**): ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm): 0.72 (1H, m), 1.04 (6H, d, *J* 6.3 Hz), 1.15 (1H, m), 1.61–1.72 (4H, m), 1.97 (1H, br d), 2.74–3.00 (6H, m), 3.20–3.25 (3H, m), 3.56 (2H, s), 4.51 (1H, m), 6.45 (2H, m), 6.84 (2H, t, *J* 8.8 Hz), 7.40 (3H, m), 7.46 (2H, d, *J* 6.5 Hz), 7.77 (1H, dd, *J* 8.0, 1.5 Hz), 8.62 (1H, dd, *J* 4.8, 1.5 Hz). MS: (ES^+) 502 (MH^+).