Identification of Structurally Diverse Growth Hormone Secretagogue Agonists by Virtual Screening and Structure−Activity Relationship Analysis of 2-Formylaminoacetamide Derivatives

Miyuki Shoda,† Takeo Harada,*,† Yuji Kogami,† Ryuichi Tsujita,† Hirotada Akashi,† Hiroyuki Kouji,† Florence L. Stahura,‡ Ling Xue,‡ and Jürgen Bajorath*,‡,§

Laboratory for Medicinal Chemistry, Institute for Life Science Research, Asahi Kasei Pharma, 632-1 Mifuku, Ohito, Tagata, Shizuoka, Japan, AMRI Bothell Research Center, 18804 North Creek Parkway, Bothell, Washington 98011, and Department of Biological Structure, University of Washington, Seattle, Washington 98195

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Two molecules with known growth hormone secretagogue (GHS) agonist activity were used as templates to computationally screen ∼80000 compounds. A total of 108 candidate compounds were selected, and five of them were found to be active in the low-micromolar range in both cell-based and direct binding assays. These compounds were structurally diverse and significantly differed from known GHS agonists. The most active compound was subjected to SAR evaluation, which slightly increased its potency and identified molecular regions important for specific GHS agonist activity.

Introduction

Growth hormone secretagogue (GHS) controls the release of growth hormone (GH) from somatotrophs in the pituitary gland.[1] In therapeutic applications, GHS agonists are capable of replacing direct administration of GH for treating various symptoms caused by GH deficiency such as growth failure. Adults with GH deficiency may suffer from osteopenia, decrease of muscle strength, high cholesterol levels, and other diseases.[2,3] GH administration is also thought to be effective in the treatment of various other symptoms such as HIV-related effects, osteoporosis,[4,5] and obesity.[6] Several peptide (GHRP-6,[7] GHRP-1,[8] GHRP-2,[9] hexarelin[10]) and non-peptide (SM-130686 (**T1**),[11] WO-0185695 (BMS, **T2**),[12] CP-424391,[13] MK-0677,[14] NN-703[15]) agonists have been reported (Figure 1). In this study, we have applied two computational screening approaches,[16] similarity searching and cell-based compound partitioning, to identify new GHS agonists in a compound inventory. In our similarity search calculations, bit string representations of molecular structure and properties (so-called fingerprints)[16] are calculated for query and database compounds and quantitatively compared using the well-known Tanimoto coefficient (Tc)[17] as similarity metric. Molecules are regarded as similar if comparison of their fingerprints reaches a predefined threshold value of a similarity coefficient. By contrast, cell-based partitioning transforms chemical descriptor spaces into low-dimensional representations and generates segments or cells for compound classification by application of binning schemes to the axes of low-dimensional space.[16] In our approach, those axes are derived from a principal component analysis (PCA) in conjunction with systematic descriptor selection through a genetic algorithm.[18] The basic idea of cell-based partitioning is that molecules occurring in the same cell are likely to display

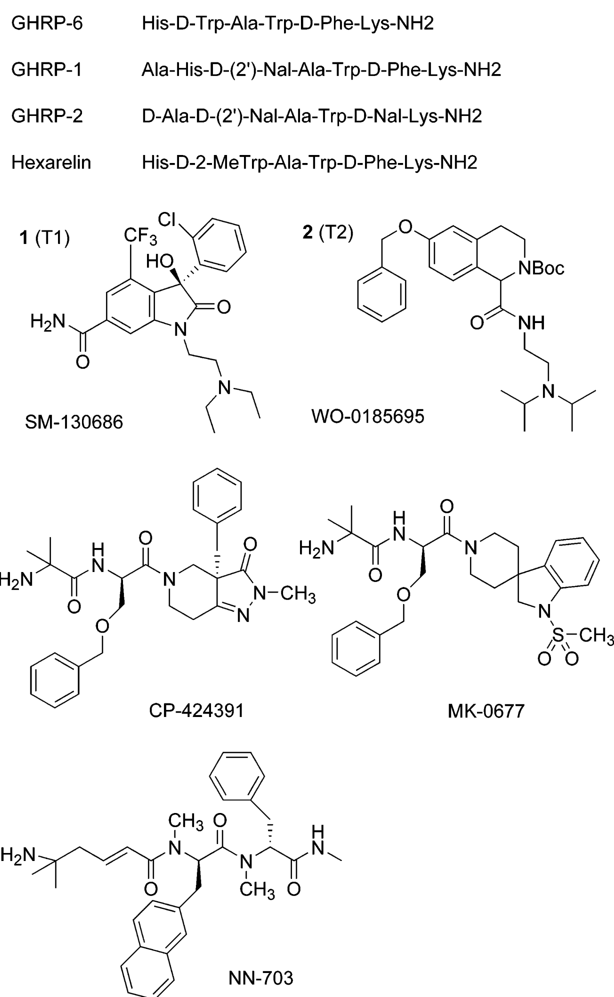

Figure 1. Reference compounds with known GHS agonist activity. Peptide agonists are represented as amino acid sequences. **T1** and **T2** were used as templates for virtual screening.

* To whom correspondence should be addressed. For T.H.: phone, +81-558-76-7076; fax, +81-558-76-5755; e-mail, harada.tt@asahi-kasei.co.jp. For J.B.: phone, +1-425-424-7250; fax, +1-425-424-7299; e-mail, jurgen.bajorath@albmolecular.com.
† Asahi Kasei Pharma.
‡ AMRI Bothell Research Center.
§ University of Washington.

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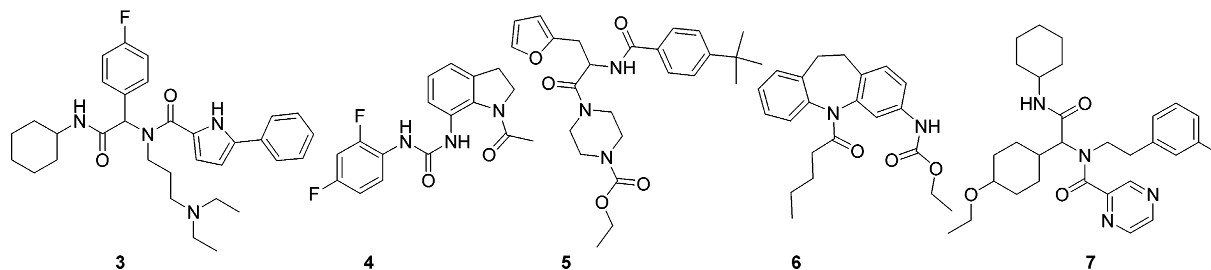


Figure 2. Structures of compounds with GHS agonist activity identified by virtual screening.

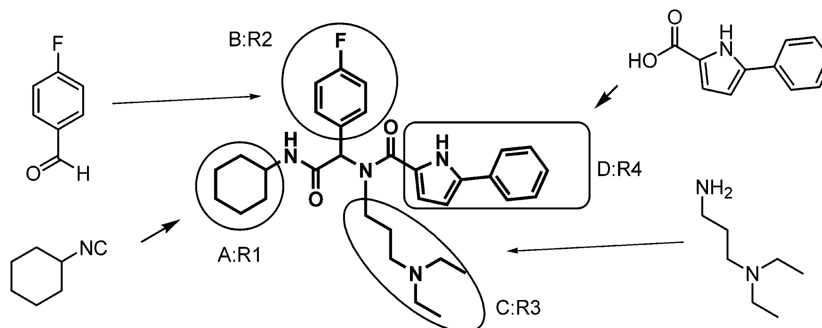


Figure 3. Reaction-based fragmentation scheme of GHS hit **3**. The compound was divided into four fragments for generation of diverse analogues.

similar biological activity. The combination of different virtual screening techniques, as applied here, is expected to increase the probability of identifying compounds having desired activity.[16] The primary goal of this analysis has been the identification of new structural classes or motifs having activity similar to that of known GHS agonists.

Results

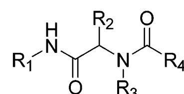
In similarity search calculations on **T1** and **T2**, the top-scoring ~100 compounds were preselected and subjected to filtering, and a total of 58 candidate compounds were chosen within the Tc interval between 0.6 and 0.8, a similarity range preferred for mini-fingerprints (MFPs).[19] In addition, on the basis of partitioning calculations, another 61 compounds were selected, 11 of which were also identified by fingerprint searching. Thus, from independent similarity search and partitioning analysis, a total of 108 candidates were selected from ~80000 database compounds for experimental evaluation.

Candidate compounds were initially tested for GHS activity in a calcium flux assay, as described in the Experimental Section. Of the 108 compounds tested for GHS agonistic activity, five were found to be active, as shown in Figure 2. Two of these compounds (**3**, **4)** were identified by similarity searching and three others (**5**–**7)** by partitioning calculations. None of these five hits were identified by both similarity searching and compound partitioning. The most active compound **3** was selected by similarity searching and displayed relative agonistic activity 57% of **T1** at 10 μM. This compound was submitted to analogue design.

For SAR analysis, **3** was considered to result from a combination of four reagent types: A, isonitril (R1); B, aldehyde (R2); C, amine (R3); D, carboxylic acid (R4) (Figure 3). This classification was based on the synthetic scheme of a four-component Ugi coupling reaction. Initially, 117 substituted amino acid derivatives similar to compound **3** were selected from in-house compound sources and tested. The core structure of these derivatives is illustrated in Table 1 that summarizes the obtained results. Only compounds with R3 = diethylaminopropyl group were found to be active. Since a morpholinopropyl group did not show any activity, despite its similarity to diethylaminopropyl, the presence of a specific amine was considered essential for GHS activity. By contrast, R2 substitutions had greater tolerance than R3. Only little information on R1 and R4 was obtained by analogue searching because of the scarcity of available variations. In the next round, R2 and R4 were derivatized. The compounds were designed so that each analogue differed only in one substituent from **3**, in order to study the substituent-specific effects. Obtained results are summarized in Table 2. According to previous results, the diethylaminopropyl group at R3 and the cyclohexyl group at R1 were conserved, with the exception of **44**. Compound **44** (R1 = substituted benzyl) was less active than compounds with cyclohexyl at the R1 position. As R2 substituents, aliphatic groups displayed higher activity than aromatic groups. Within this series, analogue **43** (R2 = butyl) was most active, as shown in Figure 4. At 10 μMn, analogue **43** was 2.8 times more active than hit compound **3**. The tertiary amine at position R3 and the aromatic moiety at R4 were identified as important pharmacophore components.

All 14 compounds that showed activity in the calcium flux assay (**3**, **8**, **11**–**13**, **28**, **31**, **34**, **37**, **40**–**44**) were tested in radioligand binding assays to confirm direct interaction with the target. As controls, two compounds that did not show calcium flux activity (**15**, **17**) were also tested. The results are reported in Tables 1 and 2 (binding curves are provided in Supporting Information) and confirm that compounds detected to be active in the initial cell-based calcium flux assays directly inhibit ligand binding to the GHS receptor. Only one of the 14 compounds (**11**) (which was weakly active in the

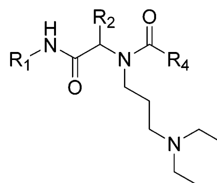
Table 1. Substitution of the Minimal Structural Core of GHS Agonist **3**[a]



compd	R1	R2	R3	R4	rel Ca^{2+} flux activity at 10 μM	radioligand binding K_i (μM)
3	cyclohexyl	4-fluorophenyl	3-diethylaminopropyl	5-phenylpyrrolyl	0.57	1.29
8	cyclohexyl	4-fluorophenyl	2-diethylaminoethyl	5-phenylpyrrolyl	0.24	1.37
9	cyclohexyl	3-indolyl	2-diethylaminoethyl	5-phenylpyrrolyl	NA	NM
10	cyclohexyl	4-(dimethylamino)phenyl	2-diethylaminoethyl	5-piperidin-1-ylmethylfuran-2-yl	NA	NM
11	cyclohexyl	4-fluorophenyl	2-diethylaminoethyl	5-piperidin-1-ylmethylfuran-2-yl	0.24	NA
12	cyclohexyl	(5-methylfuran-2-yl)methyl	3-diethylaminopropyl	5-phenylpyrrolyl	0.28	1.17
13	*tert*-butyl	methyl	3-diethylaminopropyl	5-phenylpyrrolyl	0.38	1.18
14	cyclohexyl	4-fluorophenyl	2-morpholin-4-ylethyl	5-phenylpyrrolyl	NA	NM
15	cyclohexyl	4-fluorophenyl	3-morpholin-4-ylpropyl	5-phenylpyrrolyl	NA	NA
16	cyclohexyl	4-fluorophenyl	3-morpholin-4-ylpropyl	5-morpholin-4-ylmethylfuran-2-yl	NA	NM
17	*tert*-butyl	4-fluorophenyl	3-morpholin-4-ylpropyl	5-phenylpyrrolyl	NA	NA
18	cyclopentyl	4-pyridyl	3-morpholin-4-ylpropyl	5-phenylpyrrolyl	NA	NM
19	cyclohexyl	4-fluorophenyl	4-methoxybenzyl	5-phenylpyrrolyl	NA	NM
20	cyclohexyl	methyl	4-methoxybenzyl	5-phenylpyrrolyl	NA	NM
21	cyclohexyl	4-fluorophenyl	phenyl	5-phenylpyrrolyl	NA	NM
22	cyclopentyl	4-pyridyl	phenyl	5-phenylpyrrolyl	NA	NM
23	cyclohexyl	(5-methylfuran-2-yl)methyl	benzyl	5-phenylpyrrolyl	NA	NM
24	cyclohexyl	(5-methylfuran-2-yl)methyl	cyclopentyl	5-phenylpyrrolyl	NA	NM
25	cyclohexyl	4-fluorophenyl	cyclohexyl	5-phenylpyrrolyl	NA	NM
26	cyclohexyl	methyl	ethoxycarbonylmethyl	5-phenylpyrrolyl	NA	NM

[a] NA, not active. NM, not measured. Calcium flux activity is reported relative to reference compound **T1**.

Table 2. R2 and R4 Substitutions in the Presence of the Essential Amine (at R3)[a]



compd	R1	R2	R4	rel Ca^{2+} flux activity at 10 μM	radioligand binding K_i (μM)
27	cyclohexyl	4-fluorophenyl	[5-(3,4-dimethoxyphenyl)tetrazol-2-yl]methyl	NA	NM
28	cyclohexyl	4-fluorophenyl	2-(3,4-dihydro-2*H*-quinolin-1-yl)ethyl	0.31	2.03
29	cyclohexyl	4-fluorophenyl	5-morpholin-4-ylmethylfuran-2-yl	NA	NM
30	cyclohexyl	4-fluorophenyl	4-amino-3-carbamoylisothiazol-5-yl	NA	NM
31	cyclohexyl	4-hydroxyphenyl	5-phenylpyrrolyl	0.52	1.32
32	cyclohexyl	4-ethoxyphenyl	5-phenylpyrrolyl	NA	NM
33	cyclohexyl	2,5-dimethoxyphenyl	5-phenylpyrrolyl	NA	NM
34	cyclohexyl	3,4-dimethoxyphenyl	5-phenylpyrrolyl	0.32	2.63
35	cyclohexyl	3,4,5-trimethoxyphenyl	5-phenylpyrrolyl	NA	NM
36	cyclohexyl	4-tolyl	5-phenylpyrrolyl	NA	NM
37	cyclohexyl	4-pyridyl	5-phenylpyrrolyl	1.16	0.91
38	cyclohexyl	2-furanyl	5-phenylpyrrolyl	NA	NM
39	cyclohexyl	2-thienyl	5-phenylpyrrolyl	NA	NM
40	cyclohexyl	methyl	5-phenylpyrrolyl	0.96	1.92
41	cyclohexyl	ethyl	5-phenylpyrrolyl	1.23	0.22
42	cyclohexyl	propyl	5-phenylpyrrolyl	1.37	0.87
43	cyclohexyl	butyl	5-phenylpyrrolyl	1.6	0.28
44	4-fluorobenzyl	4-fluorophenyl	5-phenylpyrrolyl	0.45	1.15

[a] NA, not active. NM, not measured. Calcium flux activity is reported relative to reference compound **T1**.

calcium flux assay) failed to show direct binding activity at detectable levels (both control compounds were also inactive). The other 13 compounds showed low-micromolar to submicromolar activity, with K_i values ranging from 2 to 0.22 μM. In general, compounds that caused the largest calcium flux were also the most active in the radioligand binding assay (for example, **41**−**43**), and there was some general correlation between the activity levels observed in these different assays. Taken together, these data confirm that the compounds identified to be active in this study are GHS ligands and elicit functional effects at the cellular level.

Discussion

Here, we have applied two different virtual screening techniques in order to select GHS agonist candidates from a medium-size compound database, and several new hits were identified and shown to be active in both

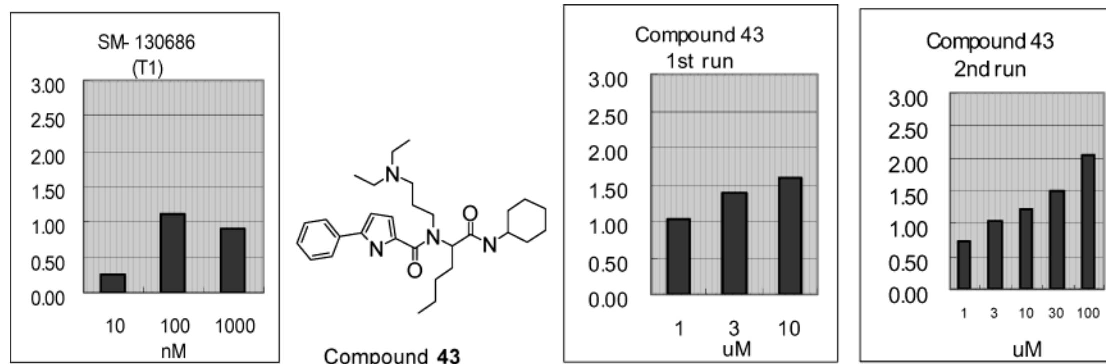


Figure 4. Ca^{2+} flux activity of SM-130686 and analogue **43**. Relative agonistic activity to reference compound **T1** is reported on the vertical axis.

direct binding and functional assays. A total of 108 candidate compounds were tested, and a hit rate of ~5% was obtained, which represents a significant improvement over random selection. The fact that none of these hits were simultaneously identified by similarity searching and partitioning emphasizes the advantages of applying different virtual screening techniques in parallel.[16] Importantly, the newly identified GHS hits were structurally diverse and significantly different from known GHS agonists. The potency of the new hits in the low-micromolar to submicromolar range is representative of what can be expected from virtual screening if a transition from one active chemical scaffold to another has successfully been made.[20] This is the case because template compounds from the (patent) literature typically represent an endpoint of a lead optimization program and not a starting point, like newly identified hits. SAR analysis of the most active hit emphasized the critical importance of a specific amino function for agonist activity of this molecule. For newly identified hits, activity in cell-based calcium flux assays corresponded to activity observed in a direct binding assay. These findings demonstrate that the compounds identified in this study act at the GHS receptor and bind to the same site as known reference and template compounds.

Conclusions

By testing a limited number of database compounds for GHS activity that were selected on the basis of virtual screening calculations, novel and structurally diverse GHS agonists have been identified. The study also demonstrates that "hit hopping" could be successfully accomplished using 2D similarity-based virtual screening methods, at least in the case of GHS agonists. Given the degree of structural diversity among template compounds and newly identified GHS hits, the findings provide further evidence for the potential of distinct chemical classes to serve as a starting point for the generation of GHS agonists.

Experimental Section

Virtual screening calculations were based on two patented compounds with known GHS activity (template **1** (**T1**) and template **2** (**T2**)) (Figure 1). A previously described, a 2D fingerprint called MFP2[19] was used to search an in-house inventory database containing ~80000 compounds for molecules similar to **T1** and **T2**. MFP2 belongs to a class of so-called minifingerprints (typically consisting of less than 100 bits) that were originally designed to recognize compounds having similar biological activity[19] and to detect a number of remote similarity relationships among receptor ligands.[21] Compounds were ranked according to their Tc values relative to **T1** and **T2**. Tc is defined as $bc/(b1 + b2 - bc)$, with b1 being the number of bits set on in fingerprint 1, b2 the number of bits set on in 2, and bc the number of bits shared by both fingerprints.[17] In parallel, PCA-based partitioning calculations were carried out after adding **T1** and **T2** as "bait" molecules to the inventory database.[18] Partitioning was performed using five different 2D descriptor combinations, as reported previously,[18] that were found to perform well in classifying compounds according to diverse biological activities. Database compounds that fell into partitions containing **T1** and/or **T2** for more than one of these descriptor combinations were selected with high priority. Compounds preselected by virtual screening were filtered for Lipinski-like criteria[22] and the presence of desired pharmacophore[23] or undesired reactive groups.[24]

Candidate compounds were initially tested using a Ca^{2+} flux assay for detection of GHS activity in A93, a cell line derived from HEK293 that constitutively expresses GHS-R1a.[11] Roughly 5.0×10^4 cells were retrieved from the culture medium, suspended, and cultivated in 96-well plates (COSTAR UV plate no. 3635) for 18−24 h at 37 °C under 5% CO_2 atmosphere. After removal of the medium, a total of 50 μL of Fura-2AM buffer (5 μM/0.2% Pluronic F-127) was added to each well and plates were incubated at 37 °C for 30 min under a 5% CO_2 atmosphere. After the sample was washed twice by the assay medium, an amount of 50 μL of assay medium was added to each of the wells that were then placed into FDSS 4000 (Hamamatsu Photonics) where 50 μL of stock solution of test compounds was automatically added. Fluorescence values were measured at 340 and 380 nm. The intensity of fluorescence indicates its agonistic activity. In control experiments on other cell lines, none of the test compounds that displayed activity on A93 were found to induce any detectable calcium flux, including Sumitomo compound SM-130686 (**T1**), which was available to us as a reference. On A93, agonistic activity of candidate compounds was evaluated as relative activity against **T1** (i.e., percentage of **T1** activity). For measurement of relative activities, the activity of **T1** at 1 μM was used as standard. The agonistic activity for **T1** is saturated at 1 μM (Figure 4). Active compounds were tested in a radioligand ([^{125}I]ghrelin (human)) binding assay following published methods.[25,26] The radioligand binding assay was conducted by MDS Pharma Services (Bothell, Washington). **T1** is reported to exhibit GH releasing activity in vivo and shows an IC_{50} value of 1.2 nM for radioligand binding assay to human GHS.[11] All analogues for SAR analysis of a newly identified GHS hit were synthesized by ASINEX (Moscow, Russia).

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Supporting Information Available: NMR spectra of compounds **3**–**45** (except for compounds **33**, **38**, **39**), ESI/UV LC spectra for compounds **33**, **38**, and **39**, and binding assays for active compounds and controls. This material is available free of charge via the Internet at http://pubs.acs.org.

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