GPCR Hits: 5HT6, CB1, D1, β2, GPR84 Generated by Fragment Libary Design

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BACKGROUND OF TARGETED FRAGMENT LIBRARIES

Fragments are important low molecular weight compounds used in systematic drug discovery methodologies for mapping protein hotspots and interaction areas. InFarmatik, Inc. has always been a company of innovative approaches in fragment based drug discovery. InFarmatik was the first company that designed fragment compounds for screening instead of using Ro3 filter for chemical inventory compounds. The concept of design was introduced to produce fragment sets with special property, resulting **targeted fragment libraries.** It was hypothesized, as in traditional screening, that these special properties could guarantee hits on some specific area. The first design aspect was to produce sterically diverse and „not flat" compounds. This led to the first fragment library characterised by unique 3D spatial properties of the library members. The **In3D Fragment Library** project was initiated in 2009 and it was first reported on FBLD 2010 (1).

The second targeted fragment library was based on selective covalent binding ability properties of acryl amides, this resulted Covalent Protein Mapping = **CPM Fragment Library.** A poster was presented on the library concept including their reactivity and selectivity on FBLD 2011 (2).

In 2011 the targeted fragment concept was extended via chemical similarity to known inhibitor/ agonist of kinase and GPCR proteins, concluding in **Kinase** and **GPCR** Fragment Libraries. **Kinase Fragment Library** concept was described and evaluated in dual kinase inhibitor project with IOTA Pharma. These results were thoroughly published in 2011 on Agrinet Conference (3), on FBLD 2012 (4) and 2013 on Practical Fragments (5).

GPCR FRAGMENT LIBRARY

GPCR Fragment Library design concept was published by InFarmatik (6) and in silico evaluation was subsequently presented by mcule.com (7). However, only limited number of in vitro assay results were produced for of selected fragments to evaluate the GPCR Fragment Library concept (6). InFarmatik, Inc in collaboration with ONTOCHEM (Germany) and Targetex (Hungary) wished to evaluate further the GPCR Fragment concept, using the following criteria:

1. mixing GPCR and Kinase fragments
2. using in silico prediction ranking
3. in parallel using structure based prediction
4. using Ca based screening on 5Ht6 and cb1
5 using reporter gene assay on human D1, human adrenergic beta2 and GP84 receptors

METHODS

Selection of the working subset (criteria #1, #2 and #3):
based on insilico screening (8) first 200 best ranked of the synthesized
compounds we selected. Inhibitor profiles we predicted using Molinspiration software (www.molispiration.com).

Description of CB1 and 5HT6 assays (TARGETEX)

CHO-K1 cells expressing stably the mitochondrially targeted aequorin (luminescent indicator) and Gα16 were used for the assay. The principle of the assay is that Aequorin (derived from Aequorea victoria) complex emits blue light while binding Ca2+ ions. The aequorin and Gα16 expressing CHO-K1 cells were transiently transfected with plasmids harboring the genes expressing CB1 or 5HT6 using Roche X-tremeGENE HP DNA Transfection Reagent. .
Cells were grown for 48 hours after transient transfection. One day before the assay the culture medium was changed to antibiotics free medium and the cells were grown for an additional 6 hours, then the cells were detached by gentle flushing with PBS/0.5 mM EDTA, recovered by centrifugation and resuspended at 1x106 cells/mL density in assay medium (DMEM/HAM's F12 D6434 with HEPES, without phenol red + 0.1% BSA + 2mM glutamine) in a Falcon tube. Coelenterazine h (Invitrogen C6780) was added at a final concentration of 5μM and the cells were incubated overnight at room temperature using constant shaking, protected from light. Before the measurement the cells were diluted 2 fold in assay medium and incubated for 60min.
The measurements were executed in Optiplate TM-96 plates (PelkinElmer 6005290) and the luminescent emission was detected by an AppliscanTM (Thermo) plate-reader. Cell suspension (45μl/well) + 5 μl antagonist (diluted in assay medium) was preincubated in the plate, the reaction was initiated by the addition of agonist (50μl) at a previously determined concentration.
The time lapse curves of receptor activation signals were recorded well by well. After detection of the base line (8sec) the device injected the agonist into one well and the change of intracellular Ca2+ level released due to receptor activation was monitored for 45 seconds. Peak luminescence signal was used for evaluation of the measurements.

Dose response curves for inhibitor standards can be seen on figure 1

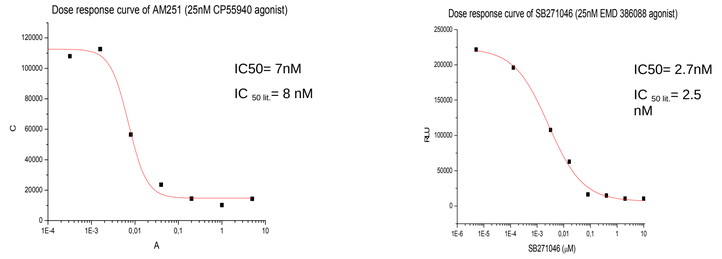
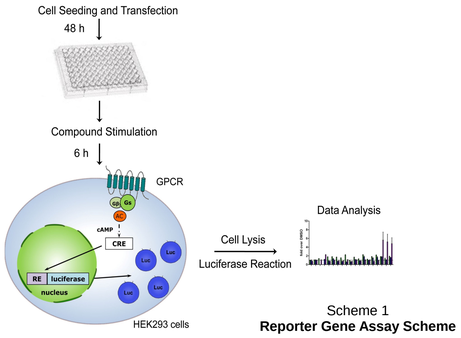


Figure 1

Dose response curves for GPCR agonists



Scheme 1
Reporter Gene Assay Scheme

Description of Reporter Gene Assays (D1,β2, GPR84 ONTOCHEM)

The schematic description of the reporter gene assays could be seen on Scheme 1

The objective of this study was to screen a subset of 60 GPCR fragment compounds towards their activity against the human dopamine D1 receptor and the human β2 adrenergic receptor, available at OntoChem GmbH

Reporter Gene Assays

Reporter gene assays were carried out using human embryonic kidney (HEK) 293 cells cultivated in DMEM/Ham's F12 (1:2) supplemented with 15% FCS and 1% L-glutamine at 37°C and 5% CO2 in a humidified atmosphere. To monitor the production of cAMP by adenylate cyclase activated through signalling of the dopamine D1 and the β2 adrenergic receptor, cells were transfected with the respective receptor plasmids and the reporter plasmid pGL4.29 (Promega, Germany). 48 hours after transfection and cell seeding in 96 well-plates, cells were stimulated with 10 μM OC compounds (10 mM stock solutions in DMSO) for 6 hours at 37°C under serum free conditions. Medium supplemented with DMSO served as non-stimulated control. Stimulations with 10 μM dopamine and adrenalin served as positive controls.
For normalization according to the cell number, 5 hours after stimulation resazurin was added to each well (50 μM final concentration) for 1 hour. Resazurin turnover and resorufin production was directly measured using a Synergy2 plate reader (BioTek,Germany). Measuring conditions were the following: end point measurement, excitation filter: 540/25 nm, emission filter: 590/25 nm. To measure luciferase activity, the stimulation medium was discarded and 30 μl of serum free medium was added to the cells. Cell lysis and detection of luciferase activity was initiated by addition of 30 μl OneGlo™ Luciferase Assay reagent (room temperature) to each well.
Cells were incubated for 5 minutes and subsequently, chemiluminescence was detected with a Synergy2 plate reader. The measured data were analysed using GraphPad Prism 5.03. Data were expressed as the ratio between luciferase activity measured in RLU and resorufin production measured in RFU, normalized to the ratio RLU/RFU of DMSO treated cells (control). Data were given as the mean ± s.e.m. of one to two experiments in triplicates.

RESULTS AND DISCUSSION

Results of the 5HT6 and CB1 assays (TARGETEX)

The primary screening has been taken at single point. The hits under 50 micromolar were screened for IC50 values.
Altogether 194 ot the 200 highest ranked synthesized fragments were tested in Ca assay of 5HT6 set and CB1. This 197 material contained 154 GPCR Fragments and 43 Kinase fragments.

IC 50 values of both 5HT6 and CB1 receptors are listed in the Table 1 below The Molinspiration pereidiction – based on literature data is shown in the middle column.

	5HT6		GPCR		CB1	
	IC50	var	predicted		**IC50**	var
			-0.39	GPCR-0019	**8,53**	1,22
GPCR-0033	**no effect**		0.12	GPCR-0033	**27,76**	7,56
GPCR-0060	**no effect**		0.09			
GPCR-0061	**26,26**	12,69	0.08	GPCR-0061	**37,06**	0,97
GPCR-0062	**31,99**	4,55	0.15	GPCR-0062	**44,91**	16,52
GPCR-0118	**11,11**	2,66	-0.04	GPCR-0118	**7,96**	5,10
GPCR-0119	**3,48**	1,37	0.39	GPCR-0119	**3,82**	1,23
GPCR-0126	**5,86**	0,78	-0.1	GPCR-0126	**25,17**	6,33
GPCR-0151	**22,19**	1,68	0.05	GPCR-0151	**51,99**	32,76
			0.08	GPCR-0163	**9,55**	1,40
GPCR-0276	**0,36**	0,11	**0.45**	GPCR-0276	**15,47**	5,13
GPCR-0277	**0,48**	0,44	**0.56**			
GPCR-0278	**0,13**	0,08	**0.51**			
GPCR-0294	**2,80**	0,34	**0.61**			
GPCR-0371	**3,74**	0,92	**0.43**	GPCR-0389	**12,89**	2,04
GPCR-0443	**2,67**	0,67	-0.1	GPCR-0443	**7,96**	1,40
GPCR-0496	**no effect**		0.09			
KIN-0179	**8,75**	0,98	0.07	KIN-0179	**2,81**	0,30
KIN-0192	**3,27**	7,70	0.09	KIN-0192	**36,29**	2,51
EL-0202	**no effect**		-0.0938			
EL-0274	**15,24**	8,62	-0.3734			
EL-0278	**no effect**		-0.5616			

Table 1
5HT6 and CB1 IC50 Ca-assay data with GPCR prediction values

Red cmpounds are false positve single point hits
Green compounds are dual or non-selective inhibitors
Black compounds are „selective" compounds

Comparing the above results to kinase screening of a similar set in PIM1/CK2 dual antagonist projekt, it looks that there is less selectivity here, than in the case of the kinases. It is also interesting that the compounds high predicted CPCR values are selective, in contrast with kinase fragments. In the case of close analogs with heavily GPCR looking fragments of GPCR-0276, 0277, 0278and 0294 there looks to be a dsitinction between the two receptor in favor of 5HT6.

Results of Reporter Gene D1, adrenerg beta2 and GPR84 assays (ONTOCHEM)

A subset of 60 compounds out of InFarmatik's GPCR fragment library was tested towards their activity against the human dopamine D1 receptor and the human β2 adrenergic receptor. Results for the reporter assay are depicted in Table 2 together with GPCR data predicted by Molinspiration software. Most of the compounds show a weak activity with approximately two- to three-fold signal over basal.

Interestingly, many compounds induced a signal transduction also in cells that only expressed YFP as control protein. This implies that these compounds most probably activate endogenous receptors present in HEK293 cells. It looks that homolog compounds are most probably behaving similarly in this experiment.

It is noteworthy, that we observed only a very weak activation of both receptors with their native ligands, adrenaline and dopamine. Dose-response curves for adrenaline and dopamine at the hβ2AR and the hD1R revealed EC50 values of 0.5 μM and 0.4 μM, respectively (data not shown). To study whether the investigated compound have an antagonistic effect on the hD1R and hβ2AR, we co-stimulated the receptors with their native ligand and 50 μM of compounds.

		dopamine D1 receptor		β2 adrenergic receptor		GPCR	YFP	
	Product_ID	fold over DMSO	S.E.M.	fold over DMSO	S.E.M.	Predicted	fold over DMSO	S.E.M.
DMSO		1.00	0.03	1.00	0.03		0.98	0.03
Dopamine		1.43	0.05				1.07	0.04
Adrenaline				1.41	0.05		3.43	0.28
OC1124	GPCR-0014	1.82	0.05	1.79	0.05	-0.24	5.62	1.85
OC1125	GPCR-0015	1.38	0.14	1.08	0.04	-0.51	5.20	1.77
OC1126	GPCR-0016	1.86	0.10	1.59	0.08	-0.68	4.80	1.36
OC1127	GPCR-0017	1.07	0.05	0.99	0.03	-0.75	5.77	2.06
OC1128	GPCR-0018	1.13	0.08	1.14	0.15	-0.82	2.23	0.51
OC1129	GPCR-0019	1.93	0.08	2.19	0.17	-0.39	5.23	1.72
OC1130	GPCR-0020	1.93	0.05	2.10	0.10	-0.1	5.46	1.55
OC1131	GPCR-0021	0.73	0.17	0.68	0.15	-0.26	5.91	2.46
OC1132	GPCR-0022	1.19	0.42	1.15	0.39	0.13	6.82	2.28
OC1150	GPCR-0051	1.28	0.05	1.25	0.14	**0.3**	1.02	0.14
OC1151	GPCR-0052	2.06	0.06	1.96	0.04	**0.36**	1.69	0.25
OC1152	GPCR-0054	1.97	0.20	2.00	0.05	**0.4**	2.07	0.07
OC1153	GPCR-0055	3.22	0.46	2.64	0.03	**0.3**	2.85	0.95
OC1154	GPCR-0056	2.45	0.12	2.33	0.18	**0.33**	2.52	0.16

Table 2
Repoter Gene Assay Data D1 and B2 data with YFP and predicted GPCR data
Green low value hits
Red higher values

The GPR84 antagonists would be valuable for teratment of inflammatory bowel diseases. The reporter gene assay identified promising hits (not showed here). The screening for GPR84 antagonist ONTOCHEM developed based on monocytic THP-1 cell line. To further increase the GPR84 expression the cell line was stimulated with bacterial LPS. The assay resulted small molcule/fragment submiclomolar GPR84 antagonists.

CONCLUSION

The Ca based assays look to be useful and robust for the first attempt in fragment screening, with acceptable error level. This assay detected hits predominantly not selective ones. It also confirmed a higher hit rate in GPCR than kinase fragments.

The reporter gene assays can also be used for GPCR fragment screening. However, this assay system has its limitations, because it is susceptible to false positive signals due to longer stimulation times. Thus, results from gene assays have to carefully validated. However, based on another assay campaign (not published) we have developed assay systems to screen for GPR84 antagonists and identified small molecules with IC50 values in the submicromolar range (patent pending)

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