

Some Unusual Pharmacology Seen in a β -arrestin Assay with the Cannabinoid CB2 receptor.

Graham Rickett

**New Screening Technologies
Pfizer Global Research & Development**

Overview

- ◆ Clinical value of CB2 receptor agonists
- ◆ Assays undertaken for this study
- ◆ Pharmacological characterisation in different assay types
- ◆ Pharmacological profile in the β -arrestin assay: details
 - ◆ Dose shift studies
 - ◆ Behaviour of Pfizer compounds
- ◆ Summary and Conclusions

Clinical Value of CB2 Agonists

- ◆ Significant efforts to develop CB2-selective agonists
- ◆ Potential clinical relevance of selective CB2 agonists mainly for pain and inflammation
 - ◆ Polypharmacological approach (one compound acting on several targets)
- ◆ Useful to investigate the CB2 receptor as an analgesic target and to modulate pain pathways in animal models
- ◆ Some relevance for other disease areas

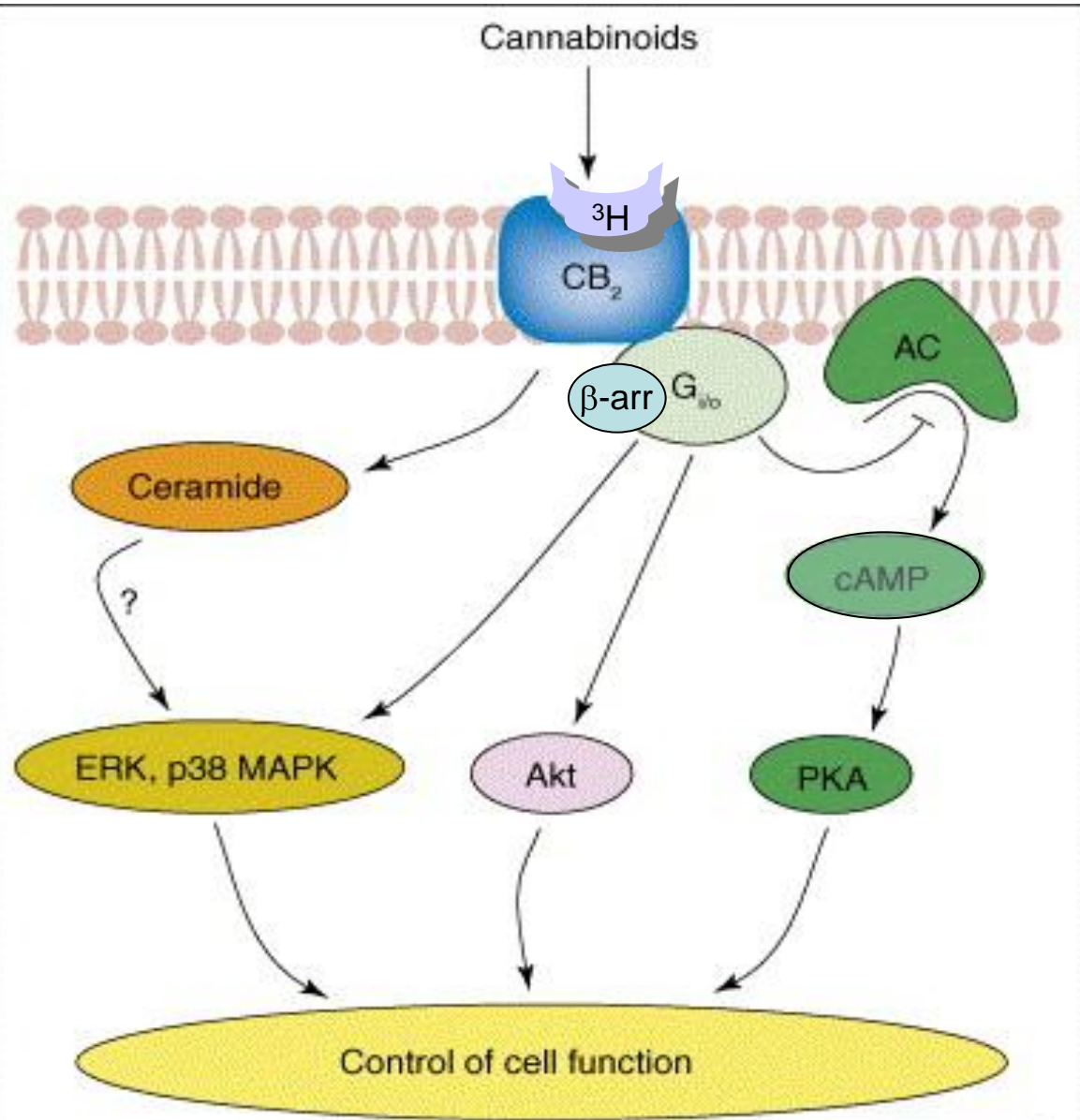
Targeting CB₂ receptors and the endocannabinoid system for the treatment of pain
Praveen Anand et al., Brain Res. Rev. 60 2009

Cannabinoids and the CB2 Receptor

- ◆ Two known Gi/o GPCRs– CB1 and CB2
- ◆ (Endo)cannabinoids act centrally (in the brain) on the CB1 receptor
- ◆ Peripheral action on the CB2 receptor not related to cannabinoid psychoactivity
- ◆ Evidence for CB2 receptors expression in human microglia, blood vessel and primary sensory neurons
- ◆ Potentially relevant receptor: orphan receptor GPR55

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CB2 Receptor Pathway



Signaling pathways coupled to the CB2 receptor

Adenylyl cyclase (AC), cAMP and protein kinase A (PKA) pathway

MAPK cascades (ERK, p38 MAPK)

Akt pathway

Pathway for *de novo* synthesis of ceramide.

Javier Fernández-Ruiz, Trends in Pharmacological Sciences 28 2007

Scope of this Study

- ◆ Investigate molecular pharmacology of CB2 active compounds in several assay types
- ◆ Evaluation of DiscoverX β -arrestin assay
- ◆ Complemented with binding and cAMP assays
- ◆ Goal to understand differences in the molecular pharmacology
 - ◆ Goal was NOT just to deliver a robust assay
 - ◆ Pharmacology is key for assay selection!

CB2 FlashBlue SPA and Filter Binding Assay

SPA 384 well Assay

- ◆ agonist
- ◆ precoupled CHO hCB2 membrane:Flashblue bead
- ◆ ^3H CP-55,490 [1nM final]
- ◆ Incubate RT for 5hr
- ◆ Read on NXT Topcount Reader

Filter Binding 96 well Assay

- ◆ agonist
- ◆ CHO hCB2 membrane
- ◆ ^3H CP-55,490 [1nM final]
- ◆ Incubate RT for 2 hr
- ◆ wash 3x with 50mM HEPES, 0.5M NaCl pH 7.4
- ◆ Incubate for 5hr
- ◆ Read on NXT Topcount Reader

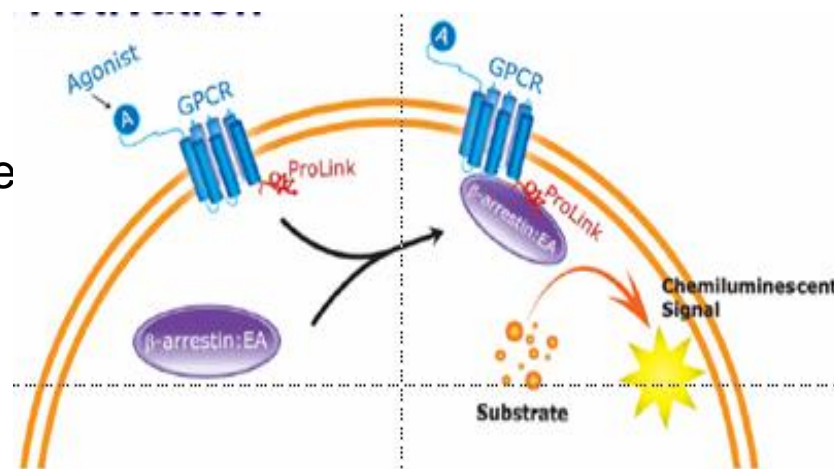
cAMP and β -arrestin Assays

384 well cAMP Assay

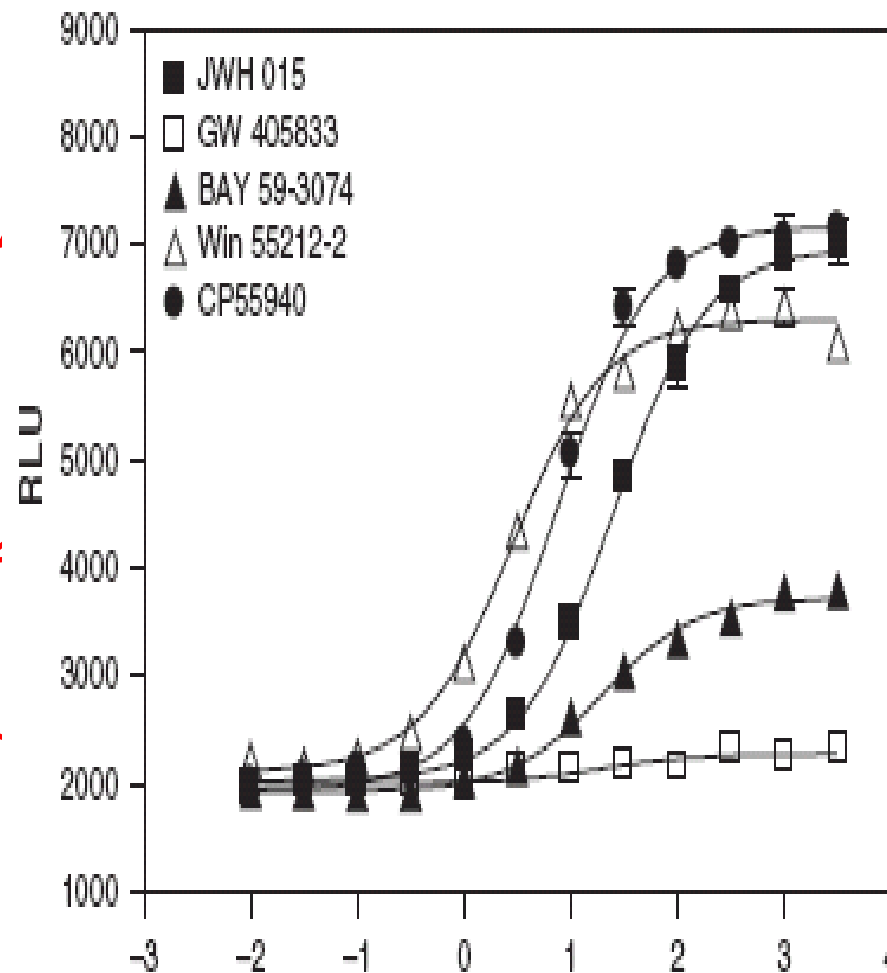
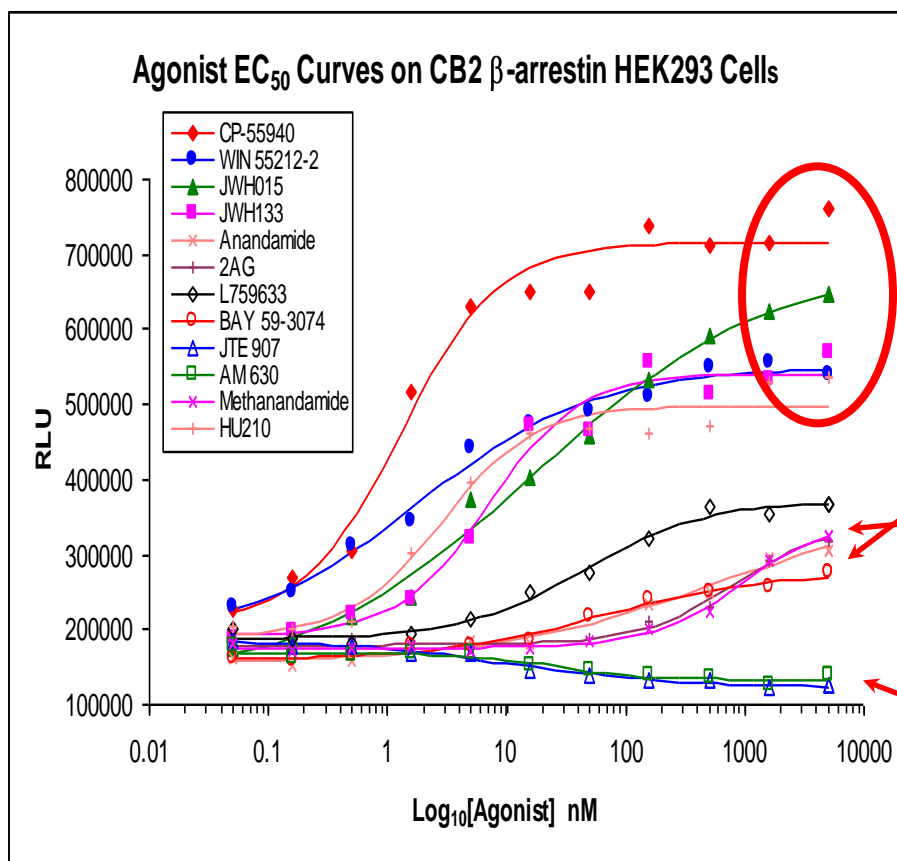
- ◆ agonist or buffer
- ◆ 20 μ M Forskolin \uparrow cAMP levels
- ◆ CHO cells incubate 1hr @ 37 C
- ◆ -80 C for 20 minutes
- ◆ HitHunter reagents added
- ◆ incubate 18hr in the dark at RT
- ◆ Read (luminescence)

384 well β -arrestin Assay

- ◆ Frozen HEK293 cells
- ◆ Remove medium, wash and replace
- ◆ agonist or buffer (90 minutes)
- ◆ detection reagent, incubate 1hr
- ◆ Read (chemiluminescence)



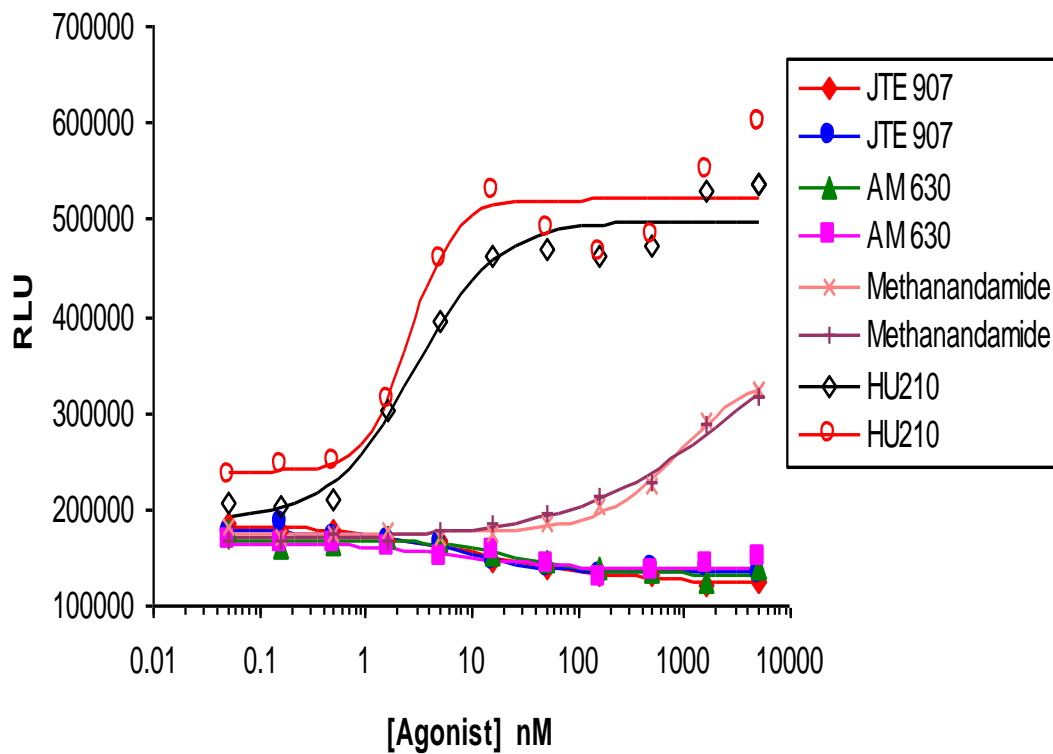
Agonist EC₅₀s in the CB2 β -arrestin Assay



Debra McGuinness et al, J. Biomol. Screen 14 (2009)

CB2 β -arrestin Assay - Summary

Agonist EC₅₀s in the CB2 β -arrestin Assay using Frozen Cells and a Change of Medium (phenol red free, serum free)



Known full agonists: Emax and EC₅₀ in β -arrestin are consistent with published data

Known partial agonist: reduced Emax in β -arrestin

Endocannabinoids: identified as partial agonists β -arrestin

Known inverse agonists show inverse agonism

Comparative EC₅₀ Data for β-arrestin, Binding and cAMP

Name	EC ₅₀ (nM) β-arrestin	Ki (nM) binding*	EC ₅₀ (nM) SPA	Ki (nM) binding	In-house cAMP	Comment
CP-55940	1.2	0.7 - 2.6	1.9	0.9	0.4	full agonist
WIN 55212-2	2.1	3.3	5.9	4.2	1.1	full agonist
JWH133	6.1	3.4	12	NT	34	full agonist
HU210	2.7	0.5	1.3	2.2	3.2	full agonist
CB 65	>5000	3.3	14	85	>8330	full agonist
Gp 1a	>5000	0.04	3.2	49	>8330	full agonist
Anandamide	272	371	299	249	673	endocannabinoid
2AG	1140	1400	1624	ND	1106	endocannabinoid
BAY 59-3074	73	46	61	21	17	partial agonist
GW405833	>5000	3.9	5.2	5.2	>10000	partial agonist
WIN 55212-3	>5000	-	510	≈5000	>8330	antagonist/inverse agonist
JTE 907	22	36	ND	19	32	antagonist/inverse agonist
AM 630	25	31	ND	16	24	antagonist/inverse agonist

* literature reported binding Kis
 ND not determined

WIN55,212-3, JTE 907, AM 630

WIN 55,212-3

Binding of [³⁵S]-GTPγS in CHO membranes showed **inverse agonism**

Sophie J. Govaerts et al. *Eur. J. Pharm. Sci.* 23 (2004)

JTE 907

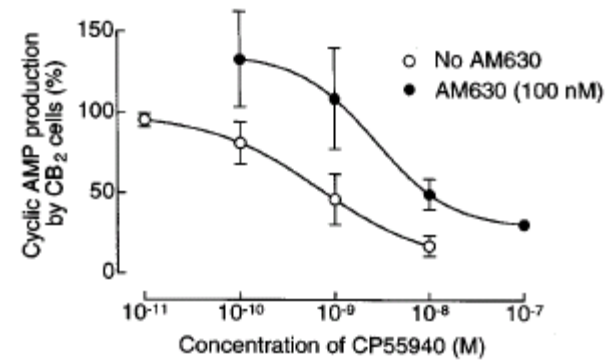
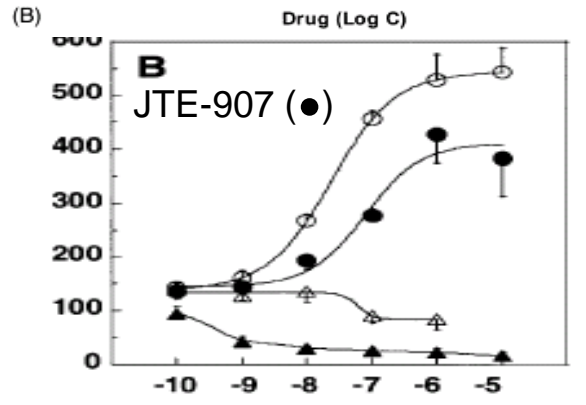
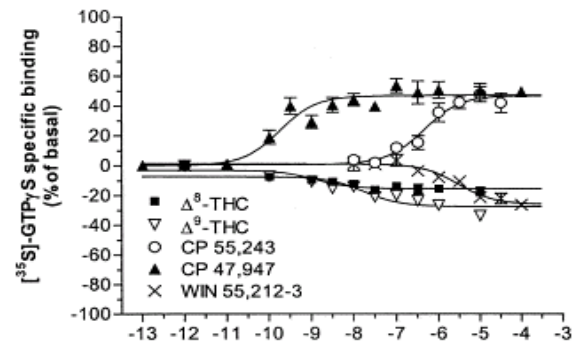
Identified as **inverse agonist** in cAMP assay in CHO hCB2 cells (forskolin)

Iwamura et al. *J. Pharmacol. Exp. Ther.* 296 (2001)

AM630

Identified as **inverse agonist** in cAMP (forskolin) and [³⁵S]-GTPγS assay in CHO hCB2 cells.

Ruth A. Ross et al. *Br. J. Pharmacol.* 126 (1999)

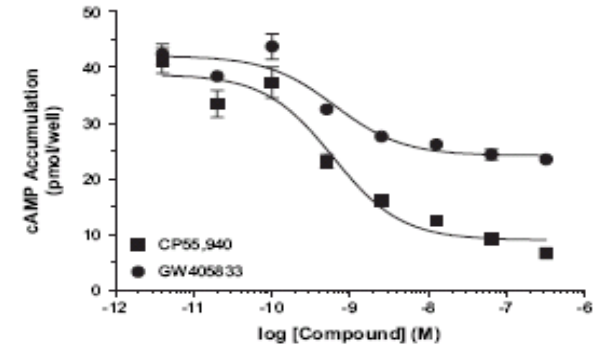


GW405833, CB65 and GP1a

GW405833

Partial agonist at human CB2 receptors; effective against nerve injury-related and incision induced pain

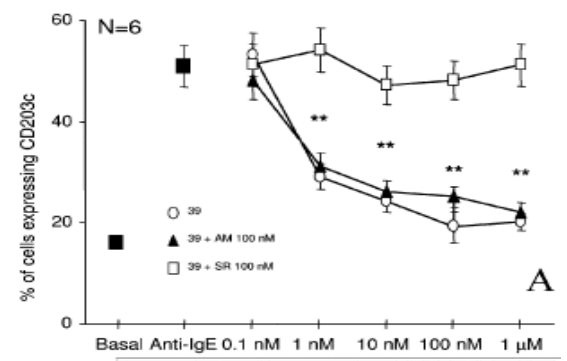
Kenneth J. Valenzano et al *Neuropharmacol.* **48** (2005)



CB 65

Activation of CB2 receptors down-regulates the immunological activation of human basophils; structural analogues of CB65 showed **agonism** in **rat spleen**

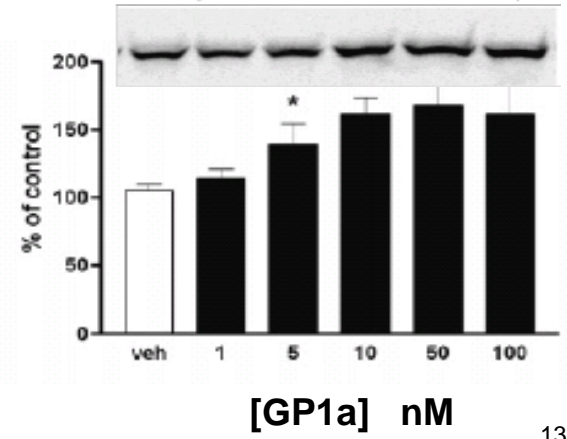
Clementina Manera et al *J. Med. Chem.* **49** (2006)



GP1a

Human promyelocytic leukemia HL-60 cells extracellular signal-related kinase (ERK) levels antagonist used to show **agonism**

Ki studies done in **mouse spleen**



Gabriele Murineddu et al *J. Med. Chem.* **49** (2006)

Antagonist pK_b Studies

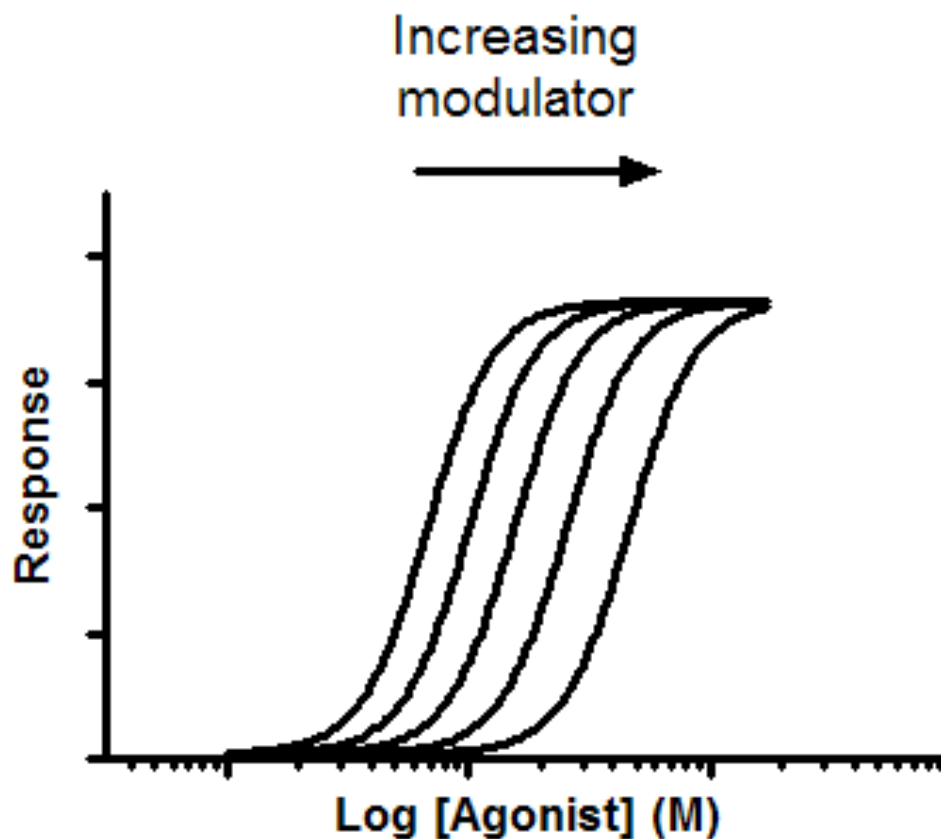
- ◆ Advantage of pK_b dose shift studies:
 - ◆ Better data quality than IC₅₀
 - ◆ Variability in [agonist] can have detrimental effect on data quality in IC₅₀ / K_i format
 - ◆ Additional mechanistic information
 - ◆ pK_b analysis at Pfizer is semi automated

- ◆ (Competitive) antagonism:
 - ◆ Is antagonism surmountable?
 - ◆ Agonist dose-response curve will be shifted to the right with the same maximum response and shape.

- ◆ How do inactive agonists behave in an β-arrestin antagonist assay?

Classical Antagonist pKb Profile

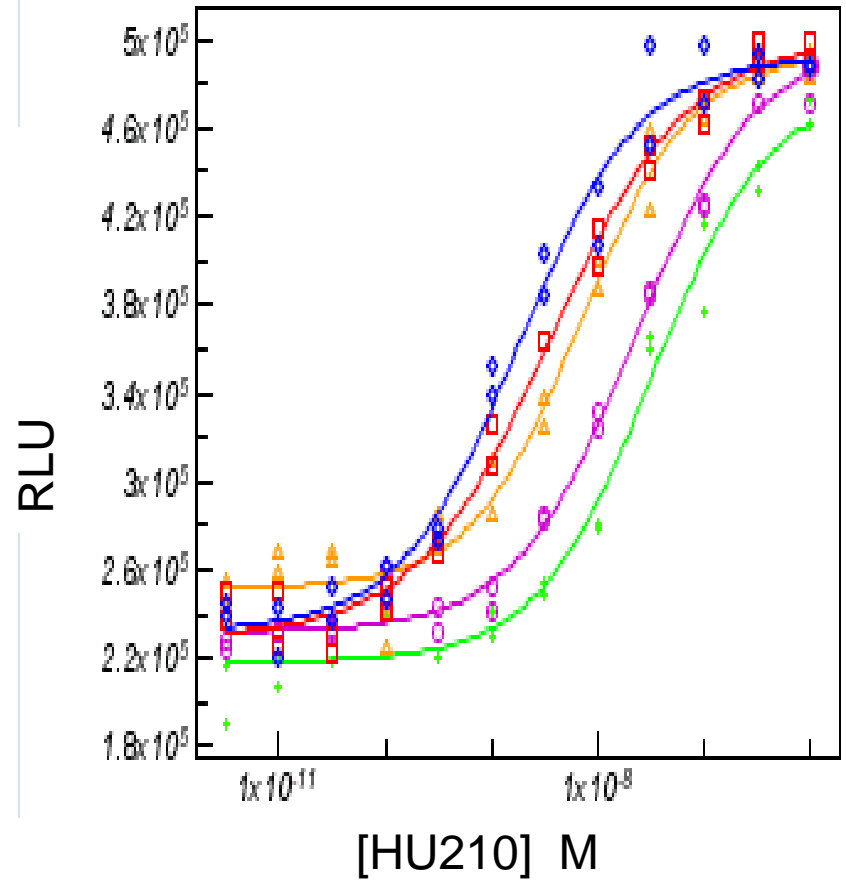
```
EC50=10^LogEC50  
Antag=1+(B/(10^(-1*pA2)))^SchildSlope  
LogEC=Log(EC50*Antag)  
Y=Bottom + (Top-Bottom)/(1+10^((LogEC-X)*HillSlope))
```



Antagonist pKbs using HU210

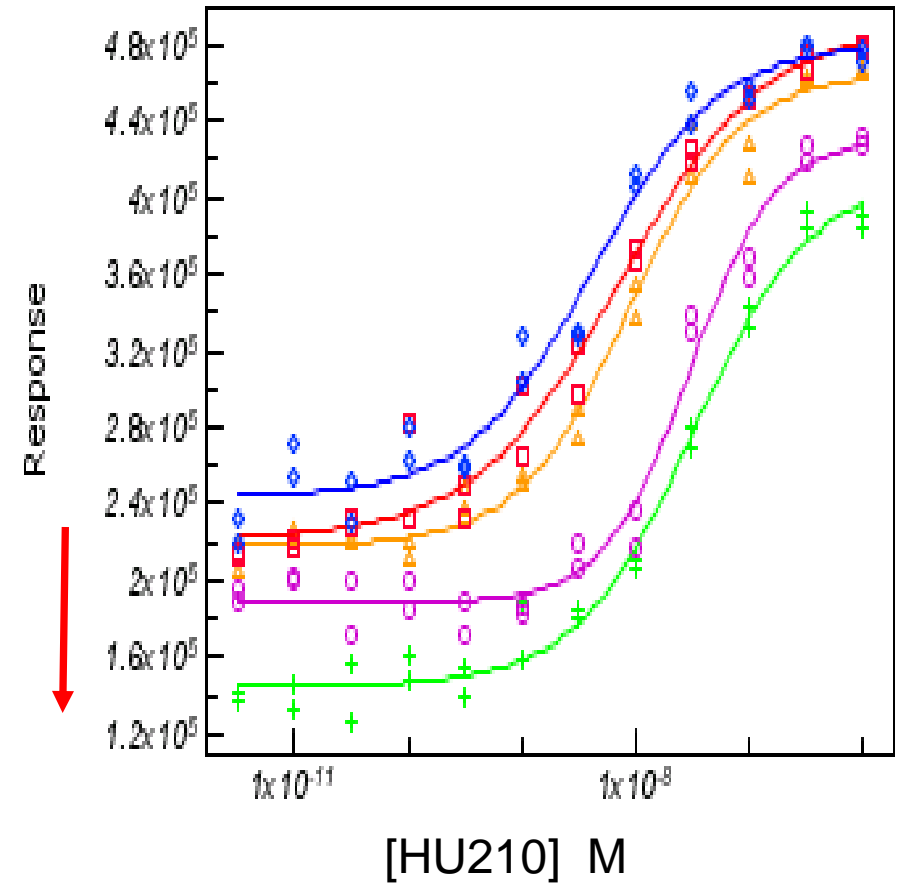
WIN55212-3 Classical Antagonist

5E-6 5E-7 0
1.58E-6 1.58E-7

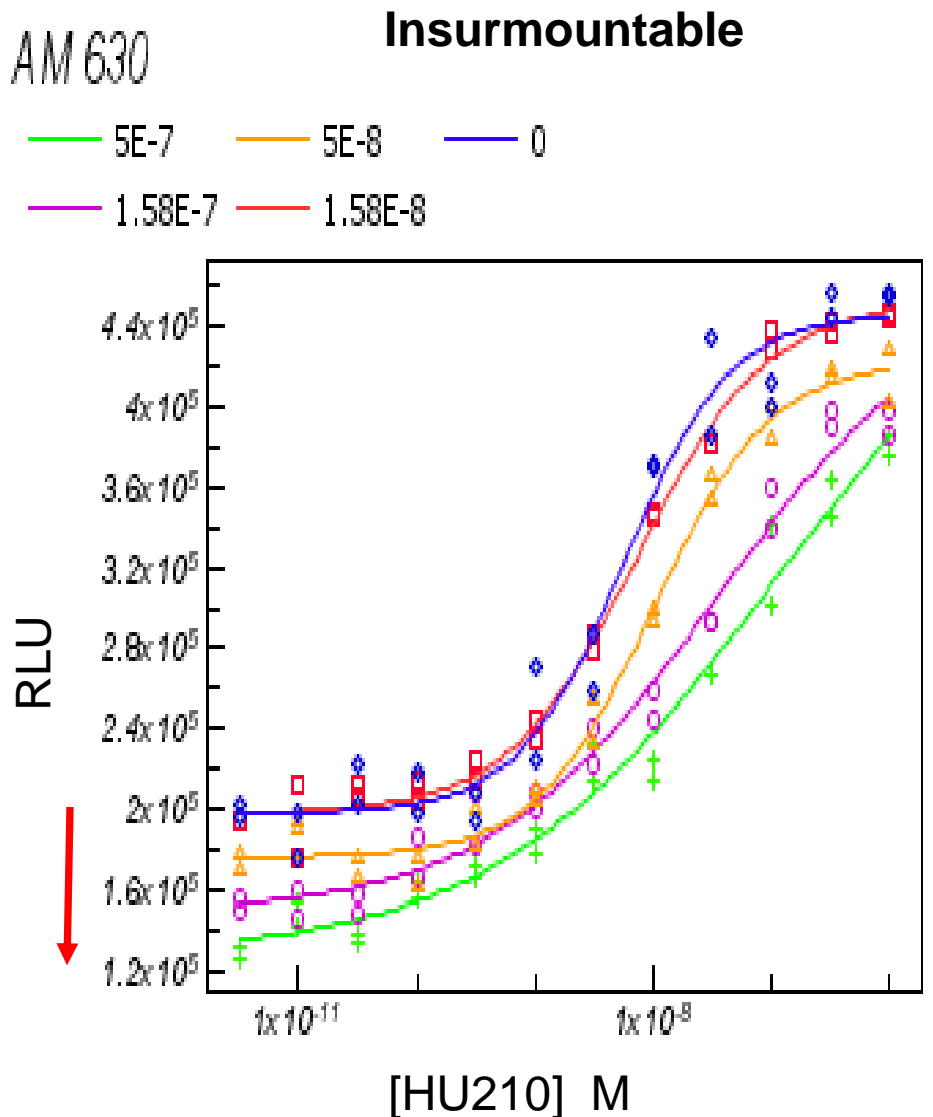


JTE 907 Insurmountable

5E-7 5E-8 0
1.58E-7 1.58E-8



Antagonist pKbs using HU210 as Agonist



Name	EC ₅₀ (nM) β-arrestin	pKb Lew/Angus	Kb (nM) *	Comment
WIN 55212-3	>5000	6.68	209	antagonist
JTE 907	22	7.56	28	insurmountable
AM 630	25	7.49	32	insurmountable

* Derived from pKb estimations

WIN55212-3:
Classical **antagonist** profile

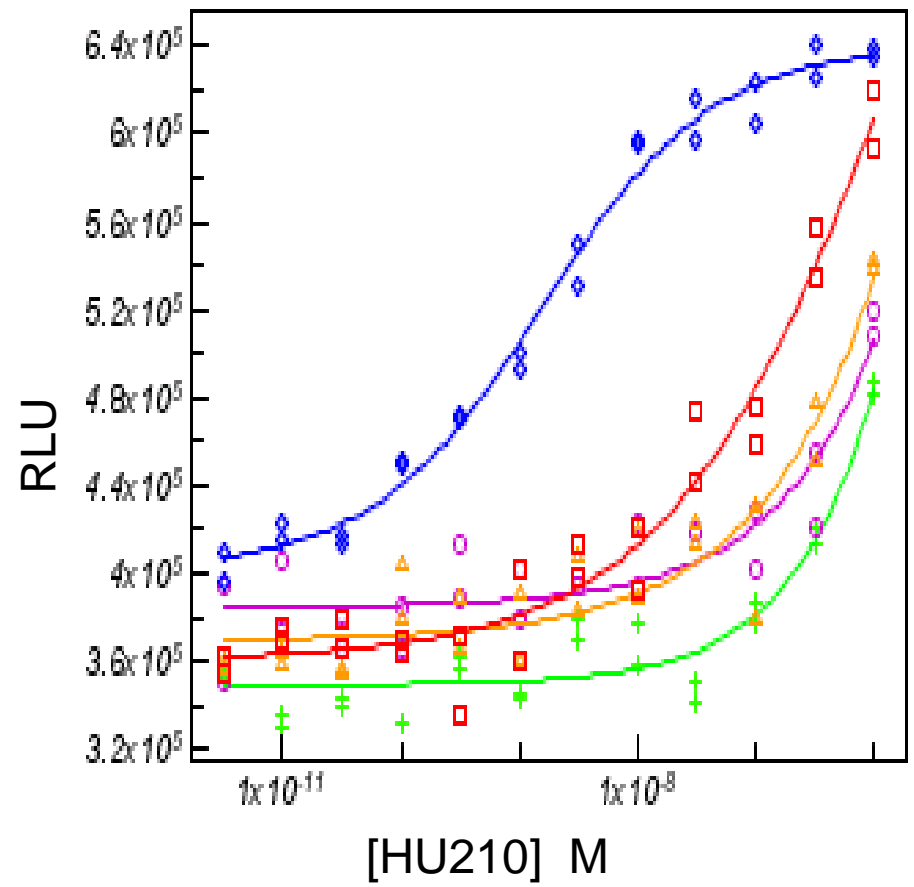
JTE907 and AM630
Decreased in agonist activity
compatible with inverse agonism

Literature Agonists in an Antagonist pKb Format (HU210 Agonist)

GW405833

Classical Antagonist

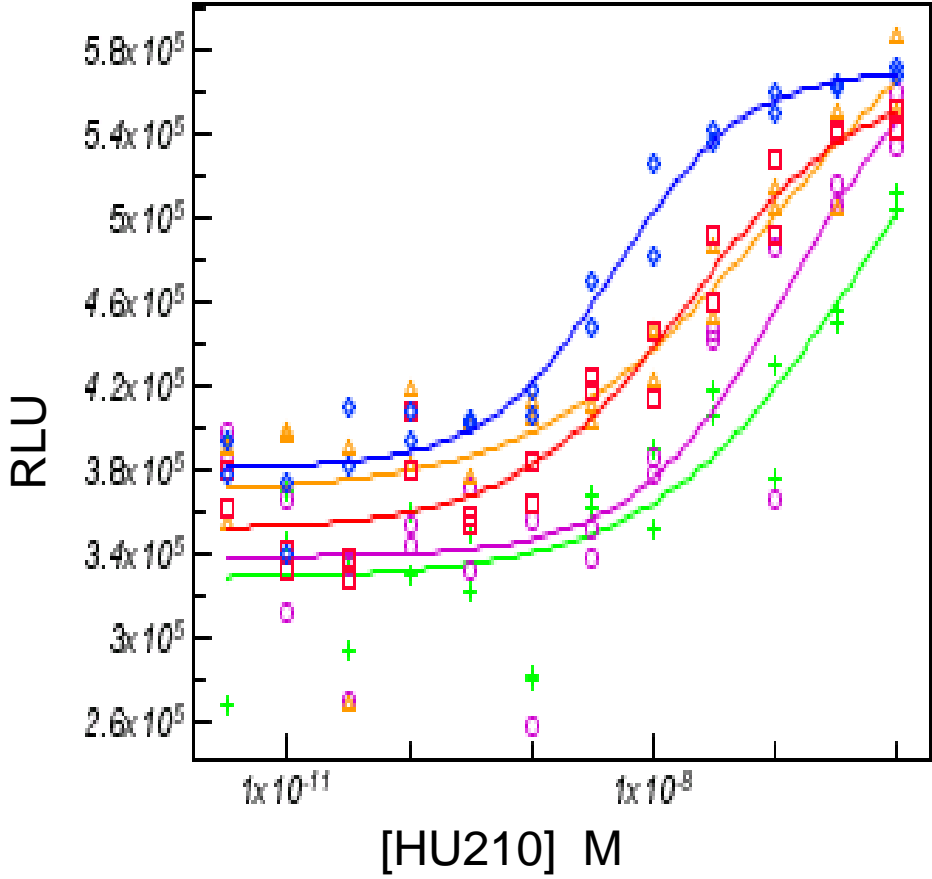
5E-6 5E-7 0
1.58E-6 1.58E-7



CB65

Classical Antagonist

5E-6 5E-7 0
1.58E-6 1.58E-7

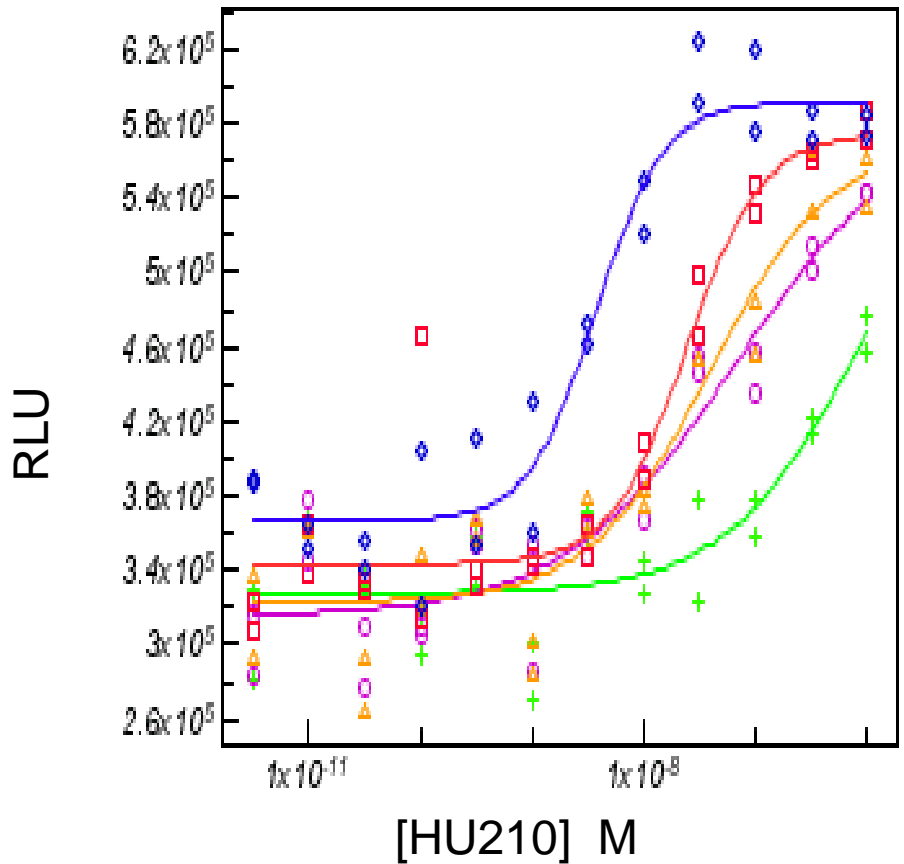


Literature Agonists in an Antagonist pKb Format (HU210 Agonist)

Gp 1a Antagonist?

— 5E-6 — 5E-7 — 0
— 1.58E-6 — 1.58E-7

Name	EC ₅₀ (nM)	pKb	Kb (nM)	Ki	Comment
	β-arrestin	Lew/Angus	*		
GW405833	>5000	8.97 [†]	1.07 [†]	5.2	antagonist
CB65	>5000	7.36	44	85	antagonist
GP 1a	>5000	7.36	44	49	antagonist

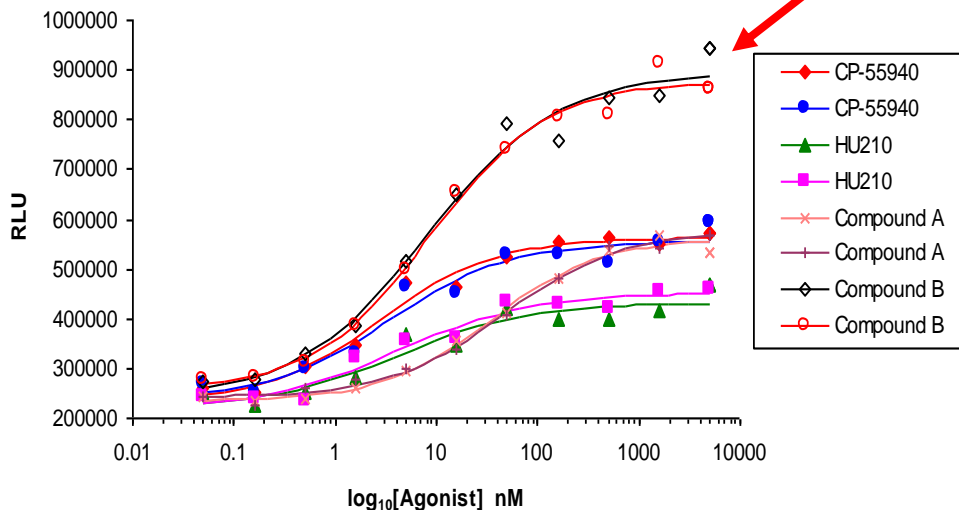


* Derived from pKb estimations † GraphPad Prism

GW 405833, CB65 + GP1a
 Appear as **antagonists** in b-arrestin;
Partial agonists/agonists in primary cell assay

Super Agonist Response in β -arrestin Assay

Agonist EC₅₀s in the CB2 β -arrestin Assay



Name	β -arrestin EC ₅₀ nM	E _{max} %	cAMP EC ₅₀ nM	E _{max} %
CP-55940	2.5	125	0.4	115
HU210	3.2	100	3.2	100
Compound A	36	126	66	98
Compound B	8.9	212	2.7	103

Compound B has shown super agonist responses in β -arrestin assay

Enhanced recruitment of β -arrestin through the CB2 receptor mechanism?

β -arrestin Assay Summary

Conclusion from this study and general features

- ◆ Pharmacological profile
 - ◆ Endocannabinoids show partial activity
 - ◆ Super agonist responses can be detected
 - ◆ Inverse agonists and antagonists
- ◆ Proximity to receptor
- ◆ High throughput and ease of use

Potential caveats

- ◆ β -arrestin non-recruiters cannot be detected
- ◆ Receptor C-terminal tag and β -arrestin fusion protein effects?
- ◆ Other arrestin recruitment in CB2 or both?

Summary and Conclusions

- ◆ β -arrestin assay: agreement with other assays types for most of CB2 agonists
- ◆ Some literature agonists show antagonist responses in β -arrestin format
- ◆ Some PFE compounds seem to show different levels of β -arrestin recruitment
- Differences not completely unexpected. Factors influencing pharmacology:
 - Species differences
 - Ratio of [receptor] to accessory proteins (issue of over-expression)
 - Different accessory proteins and pathways in different cellular background
 - Impact of where you measure in the pathway
- No one surrogate assay may be enough to determine the efficacy of compounds for *in vivo* translation
- Need to use good tools to benchmark against the natural receptor system in a physiologically relevant context

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