



# Shifting the ligand binding paradigm with Tag-Lite®

Drug Discovery

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March 2011





# Outline

- Long-acting muscarinic antagonists
- Technology evaluation – non-radioactive binding assay
  - compound profiling
  - kinetic interaction studies to address residence time
- Comparison of probe compounds
- Characterization of reference compounds
- Outlook



# Long-acting muscarinic antagonists

## Challenges and opportunities

- Clinically relevant slow acting antagonists e.g. Spiriva (Tiotropium bromide) and other LAMAS
- Slow acting mode important but measurement is a challenge

Compound	$t_{1/2}$ (h)	Efficacy after 24 hrs (%)
Tiotropium	27	35
Aclidinium	10.7	21
Glycopyrrolate	6.1	0



# Long-acting muscarinic antagonists

0022-3565/09/3302-660-668\$20.00

THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

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JPET 330:660-668, 2009

Vol. 330, No. 2  
152470/3498433  
Printed in U.S.A.

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## Preclinical Evaluation of Long-Acting Muscarinic Antagonists: Comparison of Tiotropium and Investigational Drugs

Paola Casarosa, Thierry Bouyssou, Sabine Germeyer, Andreas Schnapp, Florian Gantner,  
and Michael Pieper

*Department of Pulmonary Diseases Research, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany*

Received February 16, 2009; accepted May 26, 2009

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British Journal of Pharmacology (2006) 148, 927-937

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[www.nature.com/bjp](http://www.nature.com/bjp)

## Quantifying the association and dissociation rates of unlabelled antagonists at the muscarinic M<sub>3</sub> receptor

\*<sup>1</sup>Mark R. Dowling & <sup>1</sup>Steven J. Charlton

<sup>1</sup>Novartis Institutes for Biomedical Research, Wimblehurst Road, Horsham, West Sussex RH12 5AB



# Residence time

## OPINION

### Drug–target residence time and its implications for lead optimization

Robert A. Copeland\*, David L. Pompliano<sup>†</sup> and Thomas D. Meek<sup>‡</sup>

NDDR Vol5, 2006, 730ff

Table 2 | Examples of the dissociative half-lives of drugs and drug candidates

Target	Drug or drug candidate	Dissociative half-life	Refs
Escherichia coli dihydrofolate reductase	Trimethoprim	8 minutes	20
HMG-CoA reductase	Compactin	15 minutes	20
Chicken dihydrofolate reductase	Methotrexate	35 minutes	20
Xanthine oxidase	Allopurinol	5 hours	20
Adenosine deaminase	Deoxycytosine	40 hours	20
HSP90	Geldanamycin	4.6 hours	39
Human PNP	DADMe-Imm-H	20 minutes	20
Human PNP	DADMe-Imm-G	2 hours	20
COX2	Rofecoxib (Vioxx)	9 hours	40
Viral neuraminidase	Oseltamivir (Tamiflu)	47 minutes	41
ERBB2/EGFR	Lapatinib	5 hours	11
Human angiotensin II type 1 receptor	Candesartan	1–3 hours	21–23, 42

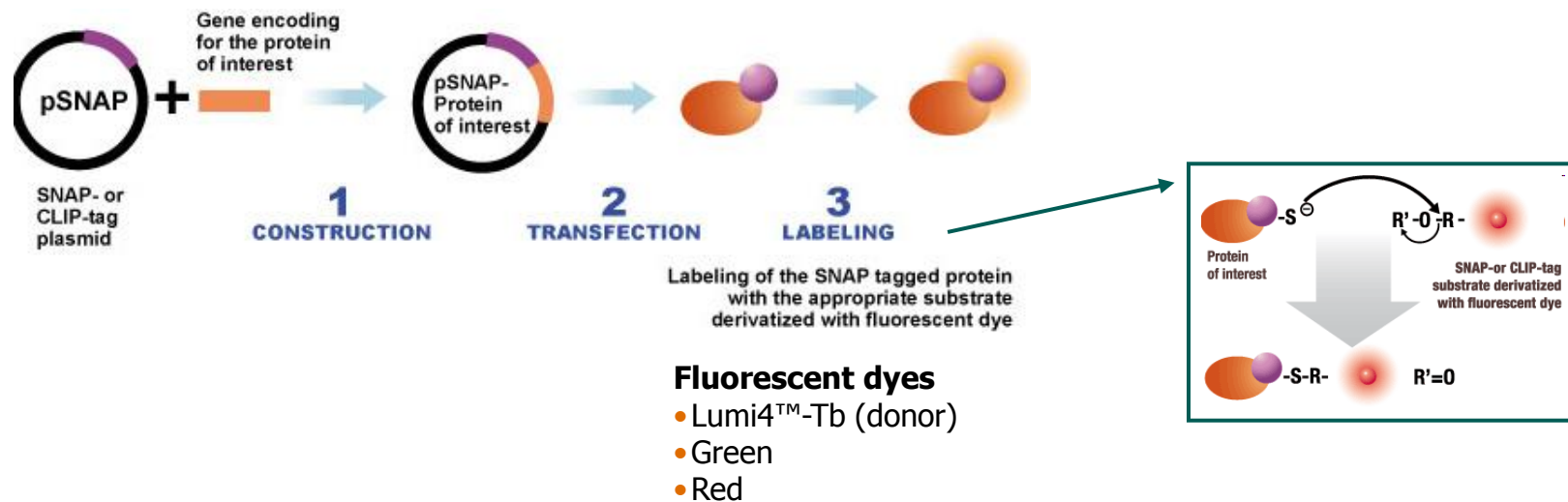
COX2, cyclooxygenase 2; EGFR, epidermal growth factor receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HSP, heat-shock protein; PNP, purine nucleoside phosphorylase.



# Tagging & labeling of surface receptors

Tag-Lite combines HTRF (TR-FRET) with SNAP/CLIP-tag technology

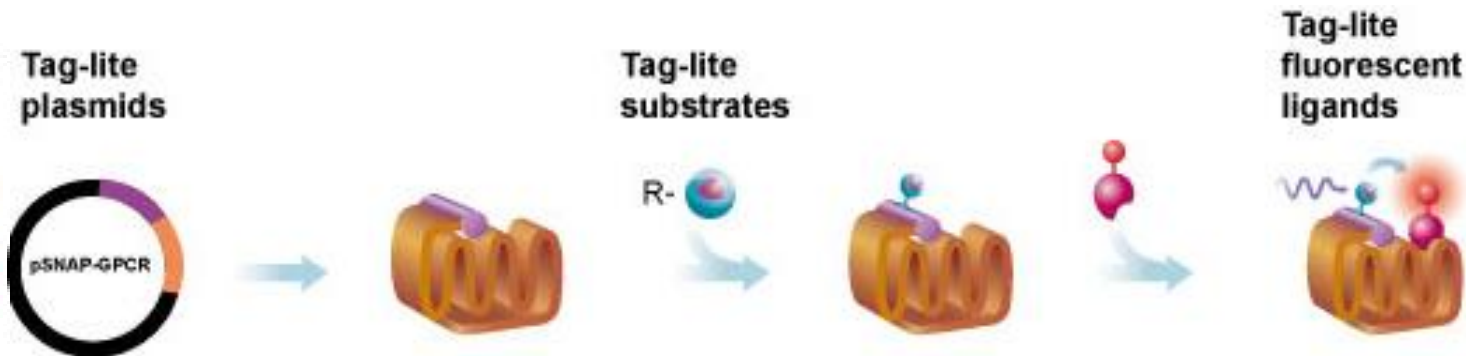
- Generation of fusion proteins with either SNAP or CLIP tags
- Labeling of fusion proteins at cell surface with synthetic HTRF dyes, which can be combined in receptor dimerization pairs or with labeled ligands



- Ideal for a wide range of applications, such as receptor dimerization monitoring, as well as ligand binding assays and second messenger assessment

# GPCR ligand binding assays

A HTRF signal is generated by interaction of the labeled ligand with labeled SNAP-tagged fusion receptor



- Unique non-radioactive ligand binding assays
- Peptidic and non-peptidic fluorescent ligands
- High specific binding
- Low non-specific binding with HTRF<sup>®</sup> technology
- Miniaturization down to < 10 $\mu$ l



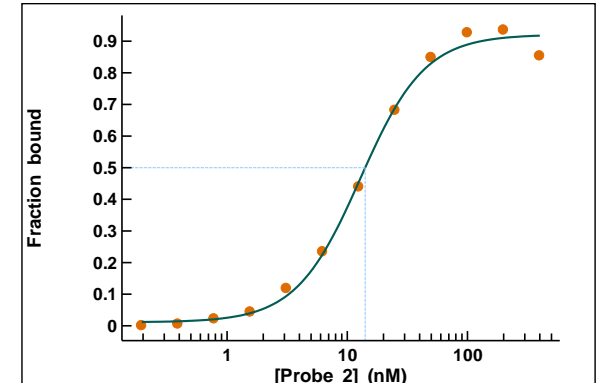
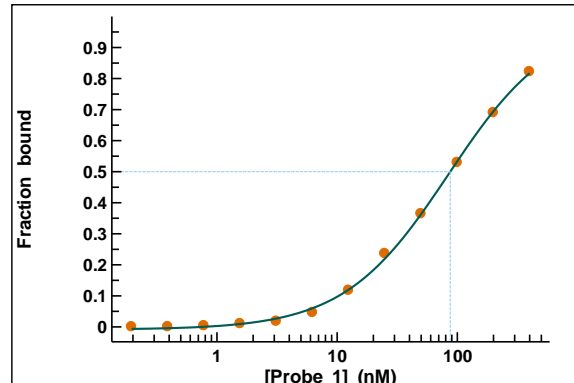
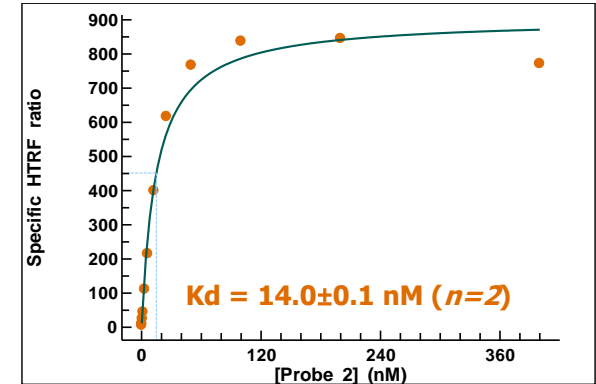
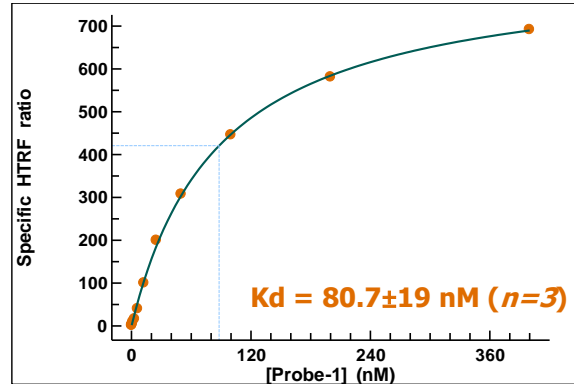
# Validation of the Tag-Lite<sup>®</sup> platform

## Muscarinic M3 receptor

- The plasmid containing SNAP-tagged muscarinic M3 receptor and/or labeled cells expressing the fusion protein are available from CisBio
- Two ligand probes for the M3 receptors were validated, both conjugated with dyes compatible with the Lumi<sup>®</sup>-Tb in HTRF
  - probe 1: Based on the muscarinic receptor antagonist Telenzepine and conjugated with red-emitting HTRF dye; provided by CisBio
  - probe 2: Based on a muscarinic receptor antagonist and conjugated to a red-emitting dye; prepared by BioFocus

# Equilibrium binding

- M3-expressing cells were labeled with Lumi4™-Tb
- 10,000 cells/well in a total volume of 20  $\mu$ l were incubated at RT with increasing concentration of probes in absence (total binding) or presence of an excess of unlabeled M3 ligand (non-specific binding)
- Signals as obtained upon probe-receptor interaction were captured by mean of HTRF ratio
- The specific HTRF signal was determined by subtraction of 'non-specific' from 'total' signal.
- The equilibrium dissociation constant ( $K_d$ ) was then calculated by the Michaelis-Menten equilibrium model



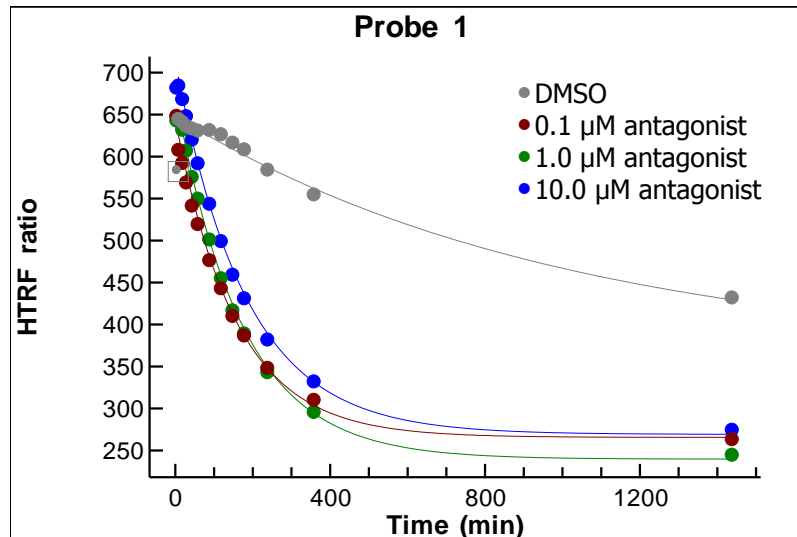
- Consistent  $K_d$  values obtained in independent experiments
- Equilibrium was achieved after 120 minutes incubation



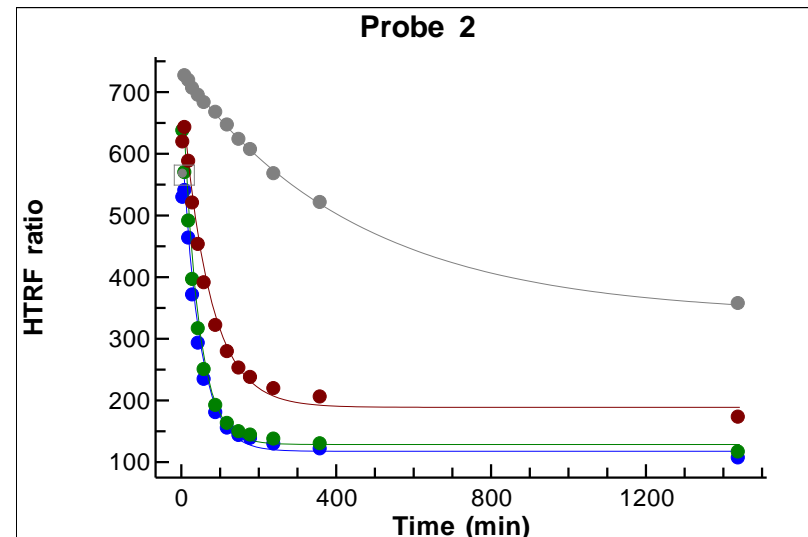
# Dissociation rate of probes

Labeled M3-cells were pre-incubated with probes for 2 h before dissociation was initiated by different concentrations of unlabelled antagonist.

Off-rates ( $k_{\text{off}}$ ) were then calculated from plotted data by using a single-phase exponential decay model.



Overall  $k_{\text{off}} = 0.0059 \pm 0.0004 \text{ min}^{-1}$  ( $n=2$ )  
 $t_{1/2} = 117 \text{ min}$



Overall  $k_{\text{off}} = 0.0239 \pm 0.004 \text{ min}^{-1}$  ( $n=2$ )  
 $t_{1/2} = 29 \text{ min}$

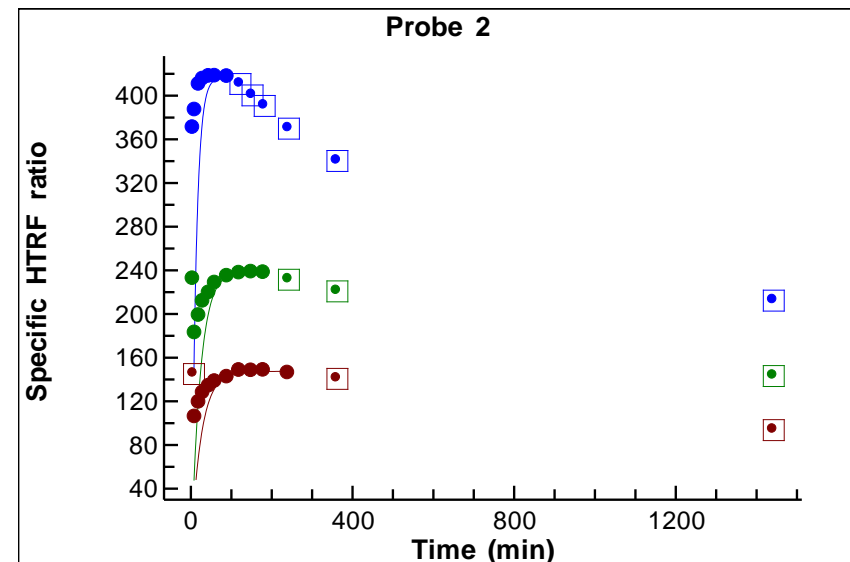
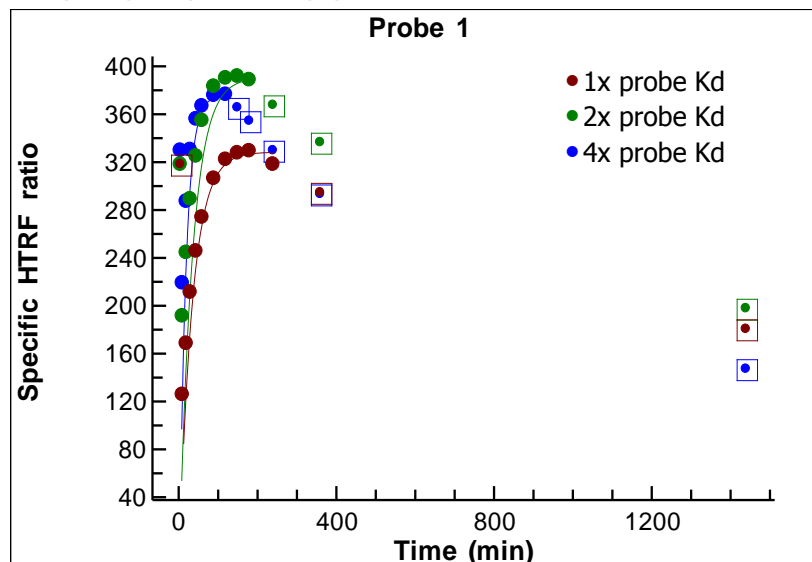
- Reproducible off rates observed



# Association rates of probes (1)

Labeled M3-cells were incubated with different concentrations of probes in absence or presence of an excess of unlabeled antagonist.

Specific binding signals were calculated, and the observed association rates ( $k_{obs}$ ) were retrieved from plotted data by using a single-phase exponential association model.



- Increase in binding-mediated signal over the first 100 min followed by a signal drop upon prolonged incubation



# Association rates of probes (2)

On-rates ( $k_{on}$ ) were calculated by using the following equation and the off-rates ( $k_{off}$ ) from separate experiments:  $k_{on} = (k_{obs} - k_{off}) / [probe]$

<b>[Probe]</b>	<b><math>k_{on} (M^{-1} min^{-1})</math> of probe 1 (n=2)</b>		<b><math>k_{on} (M^{-1} min^{-1})</math> of probe 2 (n=3)</b>	
	<b>mean</b>	<b>s.e.m</b>	<b>mean</b>	<b>s.e.m</b>
1x Kd probe	3.76E+05	7.51E+04	3.51E+06	2.46E+06
2x Kd probe	3.03E+05	1.52E+05	1.46E+06	8.41E+05
4x Kd probe	3.20E+05	1.51E+05	9.90E+05	2.07E+05
<b>Overall mean ± s.e.m</b>	<b>3.33E+05 ± 2.88E+04</b>		<b>1.99E+06 ± 1.02E+06</b>	

- Rather variable on-rates observed at different probe concentrations and in independent experiments
- Too few data points were available for determination of on-rate by plotting  $k_{obs}$  versus probe concentration (linear regression analysis, where slope =  $k_{on}$ , and intercept =  $k_{off}$ )



# Comparison of Kd value

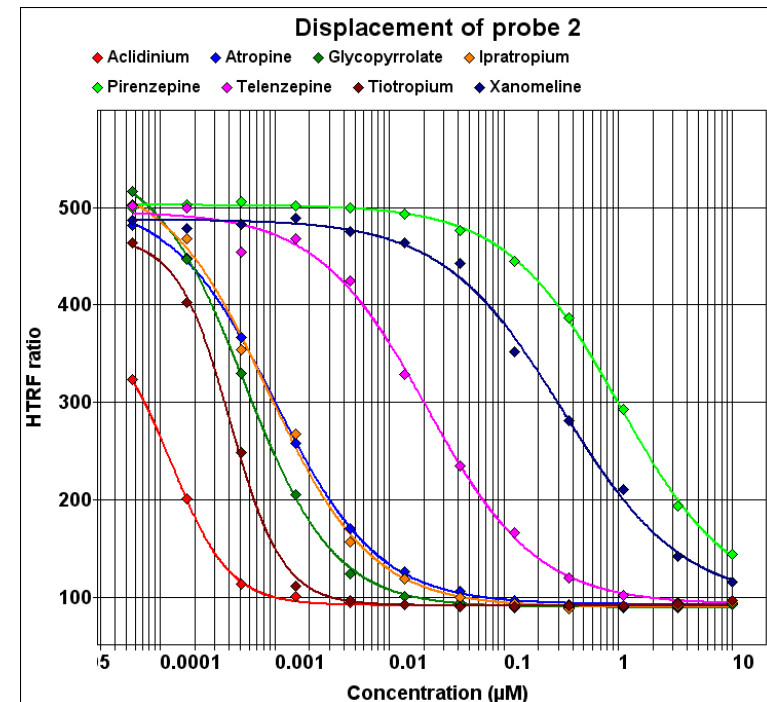
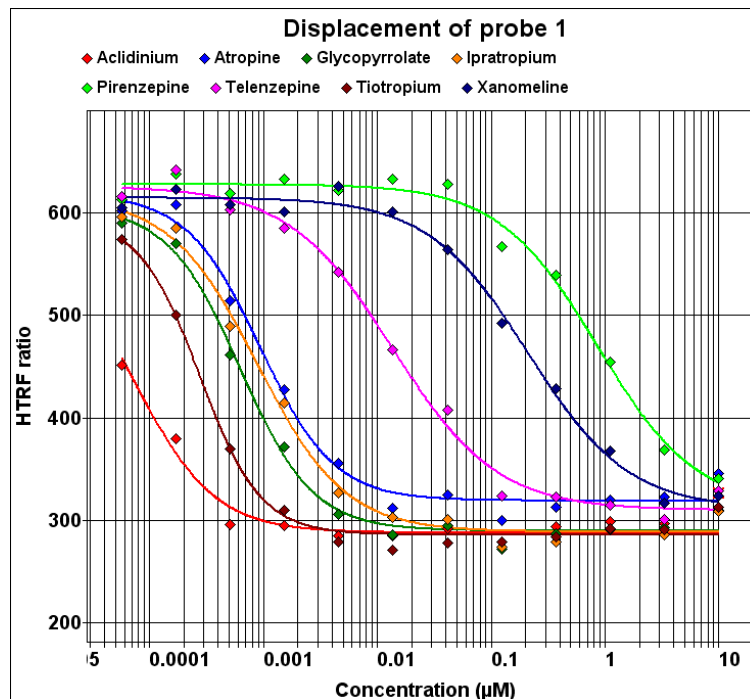
The dissociation constant Kd can be derived by following equation using the experimental on and off-rates ( $k_{on}$  and  $k_{off}$ ) :  $K_D = k_{off} / k_{on}$

	<i>Probe 1</i>	<i>Probe 2</i>
Equilibrium binding experiments (see slide 5)	80.7±19 nM	14.0±0.1 nM
Calculated from association and dissociation experiments	17.7 nM	12.0 nM

- The kinetically-derived Kd values for the probes only partially match the Kd figures from the equilibrium binding
- The high variance in the on-rate might explain the discrepancy

# Binding competition - unlabeled ligand

Labeled M3-cells were incubated with probes at their  $K_d$  and with increasing concentrations of several known M3-Ligands.



- The unlabeled ligands exhibited the same potency range and rank order for probes 1 and 2



# Comparison of Ki values

## Literature data

The observed IC50 figures from the binding competition were transformed into Ki values by using the Cheng-Prusoff equation:

$$K_i = IC_{50} / (1 + [Probe]/K_d \text{ of probe})$$

	<i>pKi</i>	
	<i>Observed</i>	<i>Literature<sup>a</sup></i>
Acridinium	10.12	9.60-10.74
Atropine	9.33	8.92-9.71
Glycopyrrolate	9.53	10.04
Ipratropium	9.35	9.58-9.76
Pirenzepine	6.36	5.57-7.03
Telenzepine	8.10	7.98 <sup>b</sup>
Tiotropium	9.74	11.02-11.10
Xanomeline	6.87	7.40

<sup>a</sup> Literature references:

1. Peralta EG et al (1987): *EMBO J*; 6; pg 3923-3929.
2. Huang F et al (2001): *APPS PharmSci*; 3; pg 1-13
3. Watson J et al (1997): *Br J Pharmacol*; 125; pg 1413-1420
4. Dowling MR and Charlton SJ (2006): *Br J Pharmacol*; pg 148; 927-937
5. Casarosa P et al (2009): *J Pharma Exp Ther*; 330; pg 660-668
6. Casarosa P et al (2009): *J Pharm Exp Ther*; 333; pg 201-206

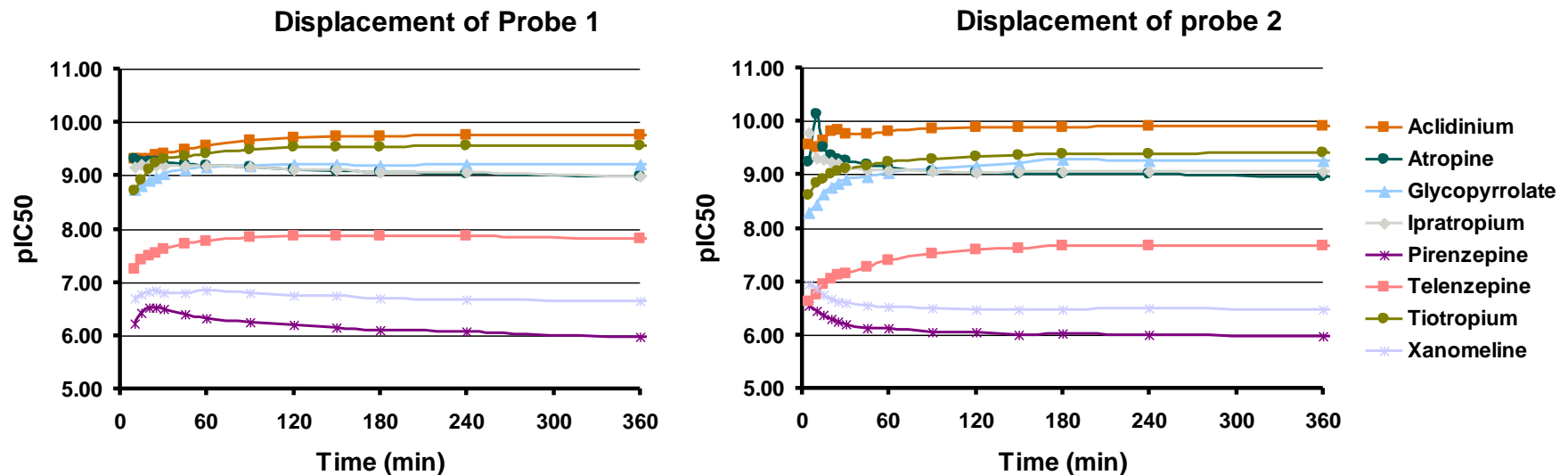
<sup>b</sup> Data from CisBio

- Overall there was a good correlation of the observed and published Ki values



# Time-dependent potency changes

Since the tested ligands differ in their binding kinetics (published data), also their potencies in binding competition may change over time until saturation is achieved.



- Similarly as reported by others (e.g. Dowling&Charlton (2006) or Casarosa et al (2009)) a time-dependent shift in potencies was observed
- The ligands with known fast dissociation rate (e.g. Atropine) showed a decrease and the ligands with known slow dissociation rate (e.g. Tiotropium) exhibited an increase in potency



# Outlook: Competition kinetic binding

## Unlabeled ligands

- Measurement of dissociation binding kinetics of unlabeled ligands
  - identify probe with fast off rate
  - use the method of Motulsky HJ and Mahan LC (1984) (reproducible  $k_{on}$  and  $k_{off}$  values for the probes are prerequisite for such investigations)
- Experimental optimization to achieve consistent  $k_{on}$  figures will be carried-out
- Technology delivers comparable  $K_d$  values compared to previous more tedious methods
  - reliable method for binding screening at equilibrium in HTS format
  - allows kinetic interaction studies
- Technology has potential for fragment based screening with GPCR targets



# Acknowledgements

Cisbio Bioassays, France for generously providing labeled M3 cells and the probe 1.

Probe 2 was kindly made available by Wesley Blackaby and colleagues at BioFocus Chesterford Research Park, Saffron Walden, UK.

Oliver Nufer for planning and execution of all experimental work and Christel Herzog for excellent technical assistance.