

# Molecular Identification and Pharmacological Characterisation of the Co-expression of M<sub>3</sub> and M<sub>5</sub> Muscarinic Receptors on the Human Astrocytoma Cell Line 1321N1

Edward Burnett<sup>1</sup>, Simon Lydford<sup>2</sup>, Diane Fellows<sup>1</sup>, James Taylor<sup>1</sup> and James Cooper<sup>1</sup>

<sup>1</sup> European Collection of Cell Cultures (ECACC), Health Protection Agency Culture Collections, Microbiology Services Porton Down, Salisbury, Wiltshire, SP4 0JG. <sup>2</sup> Molecular Devices (UK) Limited, 660 - 665 Eskdale Road, Winnersh Triangle, Wokingham, Berkshire, RG41 5TS.

## Introduction

Muscarinic G protein coupled acetylcholine receptors are widely distributed about the body and are associated with many diseases including Alzheimer's<sup>6</sup>, lung disease<sup>3</sup> and disorders of the bladder, gastrointestinal tract, salivary glands, nervous system, eye and heart<sup>1</sup>. Thus these receptors are common targets for novel therapeutics. With the increasing drive to use physiologically relevant material in drug discovery it is of interest and value to characterise native cell lines for their endogenous receptor expression with techniques ranging from PCR to functional characterisation using label-dependent and label-independent assays.

The human astrocytoma cell line 1321N1 (ECACC 86030402)<sup>8</sup> is commonly used as host for recombinant receptor expression for cell based assays. The cell line was previously characterised by the authors for its endogenous receptor expression through the exposure of the cells to the LOPAC<sup>®</sup> compound library (Sigma Aldrich) and the analysis of responses using the CellKey<sup>®</sup> (Label-Free Cellular Analysis System) ("Receptor Panning"). That study concluded that the cell line was likely to express Histamine (H1), Muscarinic and  $\beta_2$  Adrenergic receptors (<http://www.sigmaaldrich.com/sigma-aldrich/technical-documents/articles/biowire/cell-culture-collections.html>)<sup>5</sup>. Previous reports suggest that a single muscarinic receptor subtype dominates in this cell line and this is most likely to be M<sub>4</sub><sup>2</sup>, or, as now seems most commonly agreed, M<sub>3</sub><sup>4,11</sup>. However the muscarinic M<sub>5</sub>, has not been previously reported for this cell line.

The aim of this study was to explore the feasibility of interrogating a cell line's transcriptome (in the form of cDNA) with molecular techniques (PCR and sequencing) to determine the potential expressed muscarinic receptor subtypes; and subsequently confirm expression by pharmacological characterisation (cell based assay). RNA from growing cultures was extracted and reverse transcribed to cDNA. The absence of non coding sequences was verified to ensure non-carry-over of genomic DNA. The resultant cDNA was analysed using PCR primers for a panel of muscarinic receptors (M<sub>1</sub>-M<sub>5</sub>). The PCR products were sequenced and the expressed receptor subtypes were confirmed as M<sub>3</sub> and M<sub>5</sub>. Pharmacological characterisation using the FLIPR<sup>®</sup> Tetra system (intracellular Ca<sup>2+</sup> assay) and the CellKey<sup>®</sup> system (Label-Free Cellular Analysis) with a range of muscarinic ligands also suggested the presence of both M<sub>3</sub> and M<sub>5</sub> receptors. Pharmacological data were subsequently compared to a recombinant CHO-K1 cell line expressing human M<sub>3</sub> receptor (CHO-M<sub>3</sub>). In this study both the 1321N1 cells line and the CHO-M<sub>3</sub> were used as a frozen "Assay-Ready" reagent for the pharmacological characterisation of their receptors.

## Materials and Methods

### 1 Culture of 1321N1 Cells for RNA Extraction:

1321N1 cells (ECACC 86030402) were cultivated in DMEM + 2mM Glutamine + 10% Foetal Bovine Serum (FBS) at 37°C in a humidified incubator to provide pellets for RNA extraction from log-phase cultures. 3 x 175 flasks containing cultures of 6.9 x 10<sup>6</sup> cells. Control flasks were cultivated in parallel and monitored using automated live cell microscopy (IncuCyte™ (Essen Bioscience)) to ensure that cells were harvested at a mid log time point (figure 1). Images were recorded of the monolayers at point of harvest (figure 2).

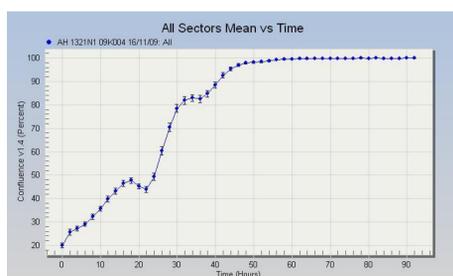


Figure 1. Example IncuCyte™ Growth Profile 1321N1 cells (ECACC 86030402) (note the unique growth profile of the cell line)

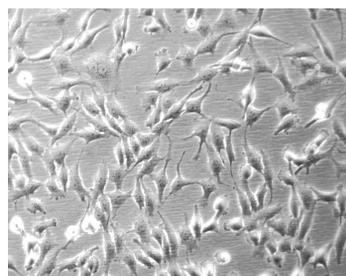


Figure 2. 1321N1 Monolayer x 100 (Log Phase)

Cultures were rapidly harvested using a Dulbecco's PBS wash at room temperature followed by warm (37°C) trypsinisation (0.25% Trypsin/EDTA). Immediately the cells detached, trypsin was neutralised using an equivalent volume of chilled (4°C) culture medium. The cell suspension was then transferred into pre-chilled (4°C) 50ml centrifuge tubes and centrifuged at 300g for 5 minutes. The supernatant was discarded and the cells re-suspended in chilled (4°C) PBS prior to a second centrifugation at 300g for 5 minutes. The supernatant was discarded and the pellet re-suspended in the residual volume before being immediately snap-frozen on dry-ice to preserve RNA and stored at -80°C until RNA extraction.

### 2 Culture of 1321N1 and CHO-M<sub>3</sub> Cells to generate Frozen "Assay-Ready" Reagent for Cell Based Assays:

1321N1 cells and the recombinant CHO-M<sub>3</sub> cell line (Inscreenex<sup>®</sup> T48-4/09 K1.3 (Human Muscarinic M<sub>3</sub> expressed in a CHO-K1 Host))<sup>12</sup> both from ECACC master cell banks were cultivated in CellStacks<sup>®</sup> (Corning<sup>®</sup>) in DMEM + 2mM Glutamine + 10% Foetal Bovine Serum (FBS) (1321N1) and Ham's F12 + 2mM Glutamine + 10% FBS (CHO-M<sub>3</sub>) at 37°C in a humidified incubator. Subculture and harvesting were effected using Dulbecco's PBS washes and 0.25% Trypsin/EDTA. Cells were cryo-preserved in 90% FBS/10% DMSO (v/v) using a rate controlled freezer to produce banks of 4 x 10<sup>6</sup> cells per vial (1321N1) and 1.3 x 10<sup>7</sup> cells/vial (CHO-M<sub>3</sub>).

### 3 Molecular Biology

Total RNA was extracted from the 1321N1 cells using the Promega Simply RNA extraction kit on a Promega Maxwell 16 automated RNA extraction system (as per the manufacturers instructions). RNA integrity was assessed initially by gel electrophoresis and then using the Agilent Bioanalyser Nanoassay. RNA purity and concentrations were measured using a Nanodrop N1000 spectrophotometer. cDNA synthesis was carried out using a one step RT-PCR kit (Qiagen #210212) using the reagents supplied by, and according to the instructions provided by the manufacturer. 1µg of RNA was used in each reaction and separate reactions were set up for using published gene-specific primers for each of the muscarinic receptors sub-types M<sub>1</sub> to M<sub>5</sub><sup>7</sup>.

Once prepared, the RT-PCR samples were incubated for 30 minutes at 50°C (reverse transcription), 15 minutes at 95°C (inactivation of reverse transcriptase and activation of Taq polymerase), then 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Finally, there was a 10 minute incubation at 72°C.

A set of  $\beta$ -actin primers designed such that they did not co-amplify processed pseudogenes<sup>9</sup>, was used as a control for the RT-PCR reaction. A potential problem in these kinds of studies involves the use of  $\beta$ -actin primers designed to amplify exons separated by an intron enabling the distinction of genomic DNA and cDNA products by their size. However, these primers were unsuitable in this study due to the presence of processed  $\beta$ -actin pseudogenes in the genome and in our experience such primers give equivalent sized (therefore indistinguishable) PCR products using genomic DNA and cDNA templates. A second PCR assay was set up using PCR primers designed to intron sequences only to test for the presence of genomic DNA carry over on the extracted RNA samples.

After PCR and RT-PCR, the amplified products were analysed by electrophoresis on 1.4% TBE gels at 100mV for 90 minutes. The identity of the PCR products was verified by sequencing using the Applied Biosystems BigDye<sup>®</sup> Terminator v3.1 Cycle- sequencing Kit and an automated Applied Biosystems model A3700 sequencer. The sequence generated was then compared to the BLAST search engine.

### 4 Pharmacology

Assay-Ready frozen CHO-M<sub>3</sub> and 1321N1 cells were thawed rapidly in a 37°C water-bath, pipetted gently into 10 ml warm growth media (Hams F-12 for CHO-M<sub>3</sub> and DMEM for 1321N1) and centrifuged for 5 minutes at 1000 rpm. Cells were re-suspended in the appropriate growth media and 50 µL/well of cell suspension was dispensed into Corning<sup>®</sup> CellBIND<sup>®</sup> black/clear base 384-well plates at a density of 15K cells/well. Plates were left on the bench for 30 min. at room temperature prior to being placed in the incubator at 37°C, 95% humidity and 5% CO<sub>2</sub> overnight.

#### (ii) FLIPR<sup>®</sup> Calcium 5 Assay Kit loading:

Dye loading buffer was prepared by dissolving the contents of one vial of FLIPR<sup>®</sup> Calcium 5 Assay Kit (Cat # R8186, Molecular Devices) completely with a final volume of 20 mL Hanks Balanced Salt Solution, 20 mM HEPES, 2.5mM probenecid (Cat # 20061, AAT Bioquest) adjusted to pH7.4. Cell plates were removed from the incubator, growth media removed and 50 mL dye loading buffer added to each well. Dye-loaded CHO-M<sub>3</sub> plates were incubated for 45 minutes at 37°C, 5% CO<sub>2</sub> and allowed to cool to room temperature for 15 minutes prior to reading on the FLIPR Tetra<sup>®</sup> system. 1321N1 plates were incubated for 60 minutes at 37°C, 5% CO<sub>2</sub> and read on the FLIPR Tetra<sup>®</sup> system at 37°C. Plates were not washed after dye loading.

#### (iii) Calcium assays on FLIPR Tetra<sup>®</sup> Instrument:

A 5X volume of agonist was prepared in HBSS buffer in 384-well polypropylene plates. Agonist (12.5 mL) was added during detection (E<sub>x</sub> 470-495 nm, E<sub>m</sub> 515-575 nm) on the FLIPR Tetra<sup>®</sup> System at optimised parameters. Antagonist was prepared at 5X concentration and added 15 minutes prior to addition of a 6X volume of EC<sub>80</sub> concentration of challenge agonist (Acetylcholine).

## Results (1) Molecular Biology:

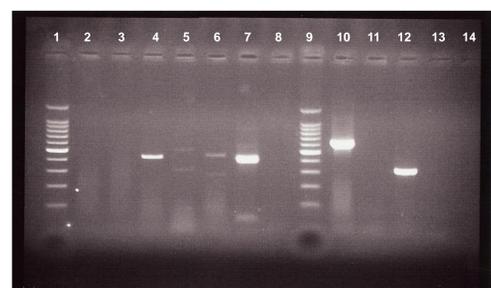


Figure 3. Results of the RT-PCR analysis of cell line 1321N1 RNA using gene-specific primers for the muscarinic receptors sub-types 1-5. Lane 1. 100bp ladder. Lane 2. Using muscarinic subtype 1 primers (M<sub>1</sub>). Lane 3. Using M<sub>2</sub> primers. Lane 4. Using M<sub>3</sub> primers. Lane 5. Using M<sub>4</sub> primers. Lane 6. Using M<sub>5</sub> primers. Lane 7. Using a second set of nested primers for M<sub>3</sub> with the first round M<sub>3</sub> product as template. Lane 8. Negative control (no template).

Controls. Lane 9. 100bp ladder. Lane 10.  $\beta$ -actin primers which do not co-amplify processed pseudogenes with 1321N1 RNA template after reverse transcription. Lane 11.  $\beta$ -actin primers with genomic DNA template. Lane 12. Intrinsic DNA primers with genomic DNA template. Lane 13. Intrinsic DNA primers with 1321N1 RNA template. Lane 14. Negative control (no template).

A clear PCR product of the predicted size is generated by RT-PCR using the primers specific for M<sub>3</sub> (figure 3). When this was sequenced and compared in a BLAST search the identity of the PCR product was confirmed as muscarinic receptor subtype 3. Two faint PCR products were generated using primers specific for M<sub>5</sub>. When this was used as a template in a second round PCR using a set of nested primers for M<sub>5</sub> a clear PCR product was generated which when sequenced was confirmed as muscarinic receptor subtype 5 (figure 4). This was not the case when the faint PCR products seen for M<sub>4</sub> were used in a second round PCR reaction as no product was formed. This M<sub>4</sub> result requires further verification.

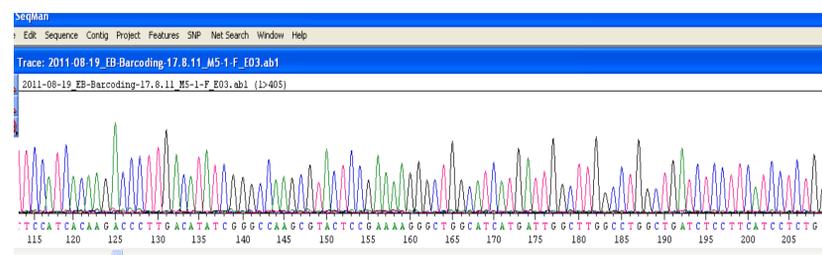


Figure 4. Partial sequence of the PCR product generated using M<sub>3</sub> primers.

## Results (2) Pharmacology

In both cell lines, FLIPR<sup>®</sup> Calcium 5 Assay Kit loaded cells were challenged with a range of muscarinic agonists and concentration-dependent responses were observed. Agonist EC<sub>50</sub> and upper asymptote estimates are shown in table 1.

Compound	1321N1 cells		CHO-M <sub>3</sub> cells	
	Agonist pEC <sub>50</sub>	Relative Asymptote ACh = 1)	Agonist pEC <sub>50</sub>	Relative Asymptote ACh = 1)
Acetylcholine	-6.4	1.00	-9.2	1.00
Oxotremorine M	-5.9	1.03	-8.6	1.02
Carbachol	-5.5	0.97	-8.1	0.98
Bethanechol	-3.7	0.66	-6.7	0.98

Table 1. Agonist data obtained from 1321N1 and CHO-M<sub>3</sub> cells using the FLIPR<sup>®</sup> Tetra system

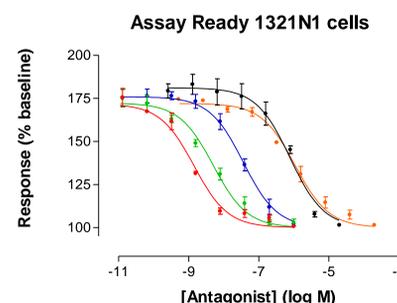


Figure 5. Antagonist IC<sub>50</sub> curves in FLIPR<sup>®</sup> Calcium 5 Kit loaded 1321N1 cells (n ≥ 4). Data were exported from ScreenWorks<sup>®</sup> software as % maximum response over baseline and analysed using GraphPad Prism<sup>®</sup>.

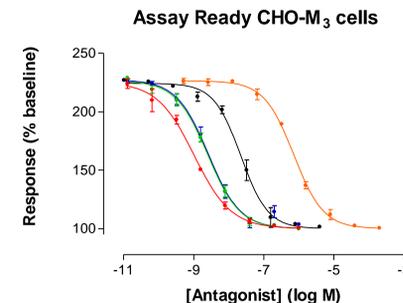


Figure 6. Antagonist IC<sub>50</sub> curves in FLIPR<sup>®</sup> Calcium 5 Kit loaded CHO-M<sub>3</sub> cells (n ≥ 3). Data were exported from ScreenWorks<sup>®</sup> software as % maximum response over baseline and analysed using GraphPad Prism<sup>®</sup>.

In subsequent studies, cells were pre-treated with one of five muscarinic antagonists before challenging with an EC<sub>80</sub> concentration of Acetylcholine. The following potency orders and IC<sub>50</sub> values were obtained;

#### 1321N1 cells (figure 5)

**Ipratropium (-8.9) > Atropine (-8.4) > Oxybutynin (-7.4) > p-FHHSiD (-6.1) = Pirenzepine (-6.0)**

#### CHO-M3 cells (figure 6)

**Ipratropium (-9.0) > Atropine (-8.6) = Oxybutynin (-8.6) > p-FHHSiD (-7.6) > Pirenzepine (-6.1)**

## Discussion

The PCR and sequencing analysis of the prepared cDNA from 1321N1 cells suggests that M<sub>3</sub> and M<sub>5</sub> subtypes are co-expressed by this cell line under the culture conditions used. Expression of the M<sub>4</sub> subtype cannot yet be fully discounted as a weak band was observed for the M<sub>4</sub> primers as can be seen in figure 3. The sequencing data confirms the identity of the expressed receptors as M<sub>3</sub> and M<sub>5</sub> (figure 4) but no sequence could be generated for M<sub>4</sub>.

We hypothesised that the co-expression of M<sub>3</sub> and M<sub>5</sub> would be observed through pharmacological characterisation (intracellular calcium assay) of the muscarinic response following exposure of the cells to agonists and antagonists and that the response may show evidence of M<sub>5</sub> expression.

The same rank order of agonist potency was observed in both 1321N1 and CHO-M<sub>3</sub> cells (see Table 1.). The absolute agonist EC<sub>50</sub> values suggest a lower receptor reserve in 1321N1 cells, and Bethanechol also appears to be a partial agonist consistent with this supposition.

In contrast, antagonist data suggests differences in the receptor(s) present in both cell lines with different rank orders of antagonist IC<sub>50</sub> estimates, see figures 5 and 6.

This antagonist data is consistent with that reported by Watson *et al*<sup>13</sup> supporting our hypothesis that 1321N1 cells express a population of muscarinic M<sub>5</sub> receptors. This is further corroborated by the molecular analysis. The Clonal CHO-M<sub>3</sub> cells express a pure population of muscarinic M<sub>3</sub> receptors as previously demonstrated<sup>12</sup>.

## Conclusions

•cDNA from 1321N1 cells proved to be a simple to use, cost effective reagent, free of contaminating genomic DNA, for the molecular analysis of the transcriptome of 1321N1 cell line. It enabled the identification of two muscarinic receptor subtypes (M<sub>3</sub> and M<sub>5</sub>).

•Subsequent pharmacological analysis using muscarinic antagonists and intracellular calcium assay (FLIPR<sup>®</sup> Calcium 5 Assay Kit) strongly support the molecular data, giving evidence that the antagonist response had some characteristics of that expected for M<sub>5</sub>.

•Further work comparing 1321N1 to a bi-cistronic recombinant cell line expressing both M<sub>3</sub> and M<sub>5</sub> would give a further insight into the pharmacological characteristics of a cell line expressing both receptors.

•Inconsistencies with previous reports<sup>2</sup> raise the hypothesis that culture conditions may influence the expression of other muscarinic subtypes in the 1321N1 cell line and that further work exploring other culture conditions, such as 3D histotypic culture would be useful.

## References

- Abrams P, Andersson K, Buccafusco JJ, Chapple C, Chet de Groat W, Fryer A.D, Kay G, Laties A, Nathanson N, Pasricha P.J, Wein A.J. Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder Br J Pharmacol. 2006 July; 148(5): 565-578.
- Ashkenazi A, Peralta E.G, Winslow J.W, Ramachandran J, Capon D.J. Functional role of muscarinic acetylcholine receptor subtype diversity Cold Spring Harb Symp Quant Biol. 1988;53 Pt 1:263-72.
- Barnes PJ. Muscarinic Receptor Subtypes: Implications for Lung Disease Thorax 1989 44:161-167
- Batty J.H, Fleming J.N, Downes C.P. Muscarinic-receptor-mediated inhibition of insulin-like growth factor-1 receptor-stimulated phosphoinositide 3-kinase signalling in 1321N1 astrocytoma cells. Biochem J. 2004 May 1;379(Pt 3):641-51.
- Cooper J. Cell Culture Collections - Exploring the Iceberg Biowire (Sigma Aldrich) Spring 2011 28-31. <http://www.sigmaaldrich.com/sigma-aldrich/technical-documents/articles/biowire/cell-culture-collections.html>
- Fisher A, Brandeis R, Bar-Ner R.H, Kligler-Spatz M, Nitan N, Sonogoe H, Marcovitch I, Pittel Z, AF150(S) and AF2678: M1 muscarinic agonists as innovative therapies for Alzheimer's disease. J Mol Neurosci. 2002 Aug-Oct;19(1-2):145-53.
- Lai J, Shoa X.M, Pan R.W, Dy E, Huang C.H, Feldman J.L. RT-PCR reveals muscarinic acetylcholine receptor mRNA in the pre-Botzinger complex. Am J Physiol Lung Cell Mol Physiol. 281: L1420-L1424, 2001.
- Macintyre E.H, Pontén J, Vatter A. E. The Ultrastructure of Human and Murine Astrocytes and Human Fibroblasts in Culture Acta Path Scand 1972;80:267
- Raff T, von der Geit M, Endemann D, Weiderholt T, Paul M. Design and Testing of  $\beta$ -actin Primers for RT-PCR that Do Not Co-amplify Processed Pseudogenes Biotechniques. 23: 456-460. September, 1997.
- Richards M.H, van Giersbergen P.L.M. Human muscarinic receptors expressed in A9L and CHO cells: activation by full and partial agonists British Journal of Pharmacology (1995) 114, 1241-1249
- Schonbrunn A, Steffen D. Endogenous GPCR List. Biomedical Computing, Inc. 2 Mar 2008. 28 Jan 2011 [www.biomedcomp.com/GPCR.html](http://www.biomedcomp.com/GPCR.html)
- Schuchert R, Lydford S, Andzinski L, Zauers J, Cooper J, Hauser H, Wirth D, May T. Rapid establishment of G-protein-coupled receptor-expressing cell lines by site-specific integration J Biomol Screen. 2011 Mar;16(3):323-31. Epub 2011 Feb 18.
- Watson N, Daniels D.W, Ford A.P, Eglen R.M, Hegde S.S. Comparative pharmacology of recombinant human M<sub>3</sub> and M<sub>5</sub> muscarinic receptors expressed in CHO-K1 cells. Br J Pharmacol. 1999 May;127(2):590-6

## Acknowledgement:

The authors are indebted to Karen Waddington of the Health Protection Agency Culture Collections for assistance with preparation and printing of this poster.