Molecular Identification and Pharmacological Characterisation of the Co-expression of M_3 and M_5 Muscarinic Receptors on the Human Astrocytoma Cell Line 1321N1

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Introduction

Muscarinic G protein coupled acetylcholine receptors are widely distributed about the body and are associated with many diseases including Alzheimer's[6], Lung Disease[3] and disorders of the bladder, gastrointestinal tract, salivary glands, nervous system, eye and heart[1]. 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 molecular PCR to functional characterisation using label dependent and label independent assays.

The Human Astrocytoma Cell Line 1321N1 (ECACC 86030402)[8] 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® compound library (Sigma Aldrich) and the analysis of responses using the CellKey® system (CDS, Label-Free Analysis) This study concluded that the cell line was likely to express Histamine (H1), Muscarinic and B-2 Adrenergic receptors[5]. Previous reports suggest that a single muscarinic receptor subtype dominates in this cell line and this is most likely to be M_4[2] or as now seems most commonly agreed, M_3[4, 11]. M_5, however 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. RNA from growing cultures was extracted and reverse transcribed to cDNA. The absence of non coding sequences was verified to ensure no carry-over of genomic DNA. The resultant cDNA was analysed using PCR primers for a panel of muscarinic receptors (M_1-M_5). The PCR products were sequenced and the expressed receptor sub-types were confirmed as M_3 and M_5. Pharmacological characterisation using the FLIPR® Tetra system (intracellular Ca^{2+}) and the CellKey® system (CDS, Label-Free Analysis) with a range of muscarinic ligands also suggested the presence of both M_3 and M_5 receptors. Pharmacological data were subsequently compared to a recombinant CHO-K1 cell line expressing human M_3 receptor (CHO-M_3). In this study both the 1321N1 cells line and the CHO-M_3 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 T175 flasks generated pellets of 6.9 x 10^6 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 (figure1). Images were recorded of the monolayers at point of harvest (figure 2).

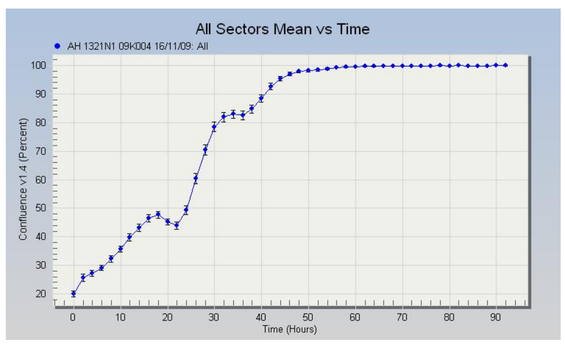

Figure 1. Example IncuCyte™ Growth Profile 1321N1 cells (ECACC 86030402) (note the unique growth profile of the cell line)

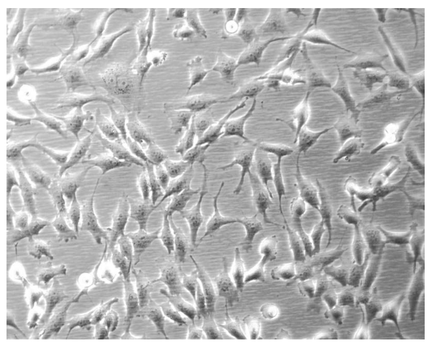

Figure 2. 1321N1 Monolayer x 100 (Log Phase)

Cultures were rapidly harvested using a Dulbecco's PBS wash at room temperature followed by warm (37°C) trypsinisation (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_3 Cells to generate Frozen "Assay-Ready" Reagent for Cell Based Assays:

1321N1cells and the recombinant CHO-M_3 cell line (Inscreenex® T48-4/09 K1.3 (Human Muscarinic M_3 expressed in a CHOK1 Host)) (Schucht), both from ECACC master cell banks were cultivated in CellStacks® (Corning®) in DMEM + 2mM Glutamine + 10% Foetal Bovine Serum (FBS) (1321N1) and Ham's F12 + 2mM Glutamine + 10% FBS (CHO-M_3) at 37°C in a humidified incubator. Subculture and harvesting were effected using Dulbecco's PBS washes and Trypsin/EDTA. Cells were cryo-preserved in 90% FBS/10% DMSO (v/v) using a rate controlled freezer to produce banks of 4 x 10^6 cells per vial (1321N1) and 1.3 x 10^7 cells/vial (CHO-M_3).

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. 1mg 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_1 to M_5[7].

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.

The control used for the RT-PCR reaction were a set of β-actin primers designed such that they did not co-amplify processed pseudogenes[9]. A potential problem in these kinds of studies involves the use of β-actin primers which are designed to exons separated by an intron, thus the genomic DNA and cDNA products can be distinguished by size. However, these primers are unsuitable due to the presence of β-actin pseudogenes in the genome and in our experience such primers give the same sized 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 were verified by sequencing using the Applied Biosystems BigDye® 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

(i) Cell handling and plating methods:

Assay-Ready frozen CHO-M_3 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_3 and DMEM for 1321N1) and centrifuged for 5 min. at 1000 rpm. Cells were re-suspended in the appropriate growth media and 50 uL/well of cell suspension was dispensed into Corning® CellBIND® 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 incubated at 37°C, 95% humidity and 5% CO_2 overnight.

(ii) FLIPR® Calcium 5 Assay Kit loading:

Dye loading buffer was prepared by dissolving the contents of one vial of FLIPR® 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 pH 7.4. Cell plates were removed from the incubator, growth media removed and 50 mL dye loading buffer was added to each well. Dye loaded CHO-M_3 plates were incubated for 45 min. at 37°C, 5% CO_2 and allowed to cool to room temperature for 15 min. prior to reading on the FLIPR Tetra® system. 1321N1 plates were incubated for 60 min. at 37°C, 5% CO_2 and read on the FLIPR Tetra® system at 37°C. Plates were not washed after dye loading.

(iii) Calcium assays on FLIPR Tetra® 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_x 470-495 nm, E_m 515-575 nm) on the FLIPR Tetra® System at optimised parameters. Antagonist was prepared at 5X concentration and added 15 min. prior to addition of a 6X volume of EC_{80} concentration of challenge agonist (Acetylcholine).

Results (1) Molecular Biology:

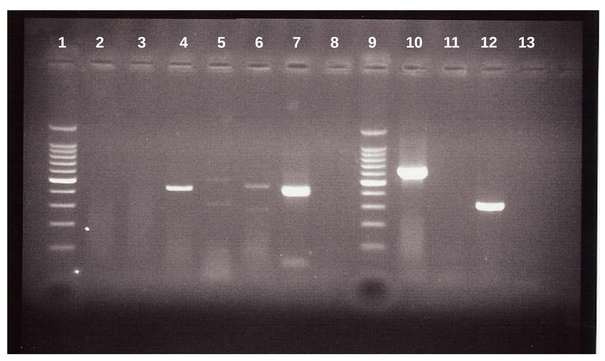

Figure 3. Results of the RT-PCR analysis of cell line 1321N1 RNA using gene-specific primers for the muscarinic receptors sub-types 1-5. Lane 1. 100bp ladder. Lane 2. Using muscarinc sub-type 1 primers (M_1). Lane 3. Using M2 primers. Lane 4. Using M3 primers. Lane 5. Using M_4 primers. Lane 6. Using M5 primers. Lane 7. Using a second set of nested primers for M5 with the first round M5 product as template. Lane 8. Negative control (no template).
Controls. Lane 9. 100bp ladder. Lane10. β-actin primers which do not co-amplify processed pseudogenes with 1321N1 RNA template after reverse transcription. Lane 11. B-actin primers with genomic DNA template. Lane 12. Intronic DNA primers with genomic DNA template. Lane 12. Intronic DNA primers with 1321N1 RNA template. Lane 13. Negative control (no template).

A clear PCR product of the predicted size is generated by RT-PCR using the primers specific for M_3 (figure 3). When this was sequenced and compared in a BLAST search the identity of the PCR product was confirmed as muscarinic receptor sub-type 3. Two faint PCR products were generated using primers specific for M_5. When this was used as a template in a second round PCR using a set of nested primers for M_5 a clear PCR product was generated which when sequenced was confirmed as muscarinic receptor sub-type 5 (figure 4). This was not the case when the faint PCR products seen for M_4 were used in a second round PCR reaction as no product was formed. This result requires further verification.

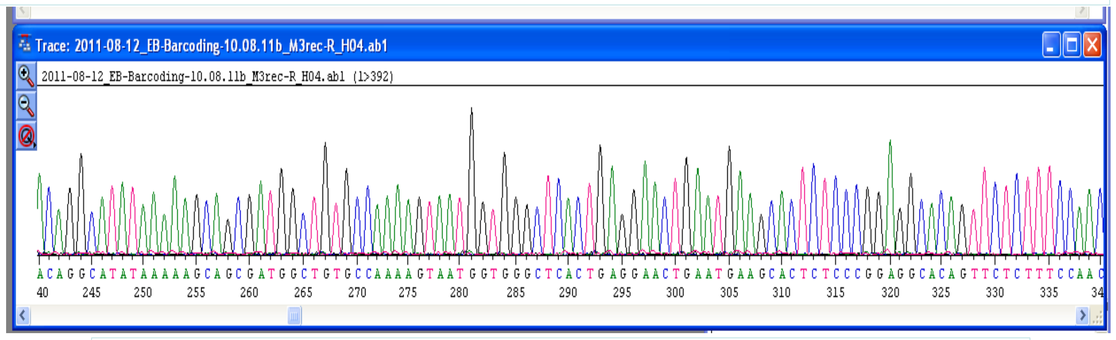

Figure 4. Partial sequence of the PCR product generated using M_3 primers.

Results (2) Pharmacology

In both cell lines, FLIPR® Calcium 5 Assay Kit loaded cells were challenged with multiple concentrations of muscarinic agonists.

Compound	1321N1 cells		CHO-M_3 cells	
	Agonist pEC_{50}	Relative Asymptote (ACh = 1)	Agonist pEC_{50}	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_3 cells using the FLIPR® Tetra system

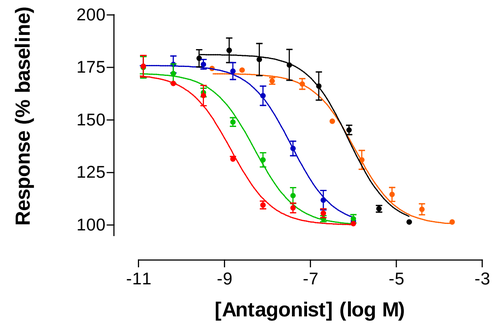

Figure 5. Antagonist IC_{50} curves in FLIPR® Calcium 5 Kit loaded 1321N1 cells (n ≥ 4). Data were exported from ScreenWorks® software as % maximum response over baseline and analysed using GraphPad Prism®.

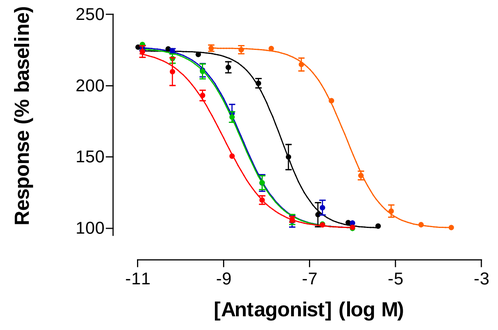

Figure 6. Antagonist IC_{50} curves in FLIPR® Calcium 5 Kit loaded CHO-M_3 cells (n ≥ 3). Data were exported from ScreenWorks® software as % maximum response over baseline and analysed using GraphPad Prism®.

The potency order and IC_{50} values obtained in 1321N1 cells (Fig. 5) was;

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

In contrast, the potency order and IC_{50} values obtained in CHO-M3 cells (Fig 6.) was;

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_3 and M_5 subtypes are co-expressed by this cell line under the culture conditions used. Expression of the M_4 subtype cannot yet be fully discounted as a weak band was observed for the M4 primers as can be seen (figure 3). The sequencing data confirms the identity of the expressed receptors as M_3 and M_5 (figure 4) but no sequence could be generated for M_4.

We hypothesised that the co-expression of M3 and M5 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_5 expression.

The same rank order of agonist potency was observed in both 1321N1 and CHO-M_3 cells (see Table 1.). The absolute agonist EC_{50} 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_{50} estimates, see figures 5. and 6.

This antagonist data is consistent with that reported by Watson *et al* [13]. Supporting our hypothesis that 1321N1 cells express a population of muscarinic M_5 receptors as supported by the molecular analysis. The Clonal CHO-M_3 cells express a pure population of muscarinic M_3 receptors as previously demonstrated [12].

Conclusions

- cDNA from 1321N1 cells proved to be a simple to use, cost effective reagent for the molecular analysis of the transcriptome for this cell line. It enabled the identification of two muscarinic receptor subtypes (M_3 and M_5).
- Subsequent pharmacological analysis using muscarinic antagonists and intracellular calcium assay (FLIPR® Calcium 5 Assay Kit) strongly support the molecular data, giving evidence that the antagonist response had some characteristics of that expected for M_5.
- Further work comparing 1321N1 to a putative bi-cistronic recombinant cell line expressing both M_3 and M_5 would give a further insight into the pharmacological characteristics of a cell line expressing both receptors.
- Inconsistencies with previous reports [2] 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.

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