Novel Pharmacological Modulators of P2X7 receptor

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis/project is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Kshitija Dhuna

Date: 18/03/19
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Academic Contribution

During the course of this thesis, the candidate’s aim was to contribute to the fields of Purinergic signalling and to characterise certain pharmacological modulators so as to attain a better insight into the P2X7 function and modulation. The candidate has contributed to the following publications and presentations:

i. **JOURNAL ARTICLES**

1. Ginsenosides act as positive modulators of P2X4 receptors
   
   **Kshitija Dhuna, Matthew Felgate, Stefan Bidula, Samuel Walpole, Lucka Bibic, Brett A. Cromer, Jesus Angulo, Julie Sanderson, Martin J. Stebbing, Leanne Stokes: Molecular pharmacology. 2018:mol.118.113696.**

2. Neonatal overfeeding by small-litter rearing sensitizes hippocampal microglial responses to immune challenge: reversal with neonatal repeated injections of saline as well as minocycline
   
   **Simone De Luca, Ivana Ziko, Kshitija Dhuna, Luba Sominsky, Mary Tolcos, Leanne Stokes, Sarah Spencer: Journal of Neuroendocrinology 29(11):e12540 · October 2017.**

3. P2X4 Receptor Function in the Nervous System and Current Breakthroughs in Pharmacology  
   **Leanne Stokes, Janice Layhadi, Lucka Bibic, Kshitija Dhuna, Samuel Fountain: Frontiers in Pharmacology 8 May 2017.**

4. Selected ginsenosides of the protopanaxdiol series are novel positive allosteric modulators of P2X7 receptors.
   
ii. **CONFERENCE PRESENTATIONS**

1. Ginsenosides cause cell death by potentiating calcium influx via P2X7 channels in a murine macrophage cell line.

   *Kshitija Dhuna, Martin J Stebbing, Ray M Helliwell, Leanne Stokes, MGPCRS-ASCEPT, Melbourne, December 2016*

2. Investigating the selectivity of ginsenoside positive modulators on P2X7

   *Kshitija Dhuna, Martin J Stebbing, Ray M Helliwell, Leanne Stokes, ASCEPT, Tasmania, December 2015*

3. Minocycline: A novel pharmacological modulator of P2X7

   *Kshitija Dhuna, Martin J Stebbing, Leanne Stokes, GAGE conference, Canberra, April 2015*

4. Minocycline modulates purinergic receptors

   *Kshitija Dhuna, Martin J Stebbing, Leanne Stokes, HDR Conference, RMIT University, Melbourne, October 2014*
# TABLE OF CONTENT

**DECLARATION** ............................................................................................................... II

**ACKNOWLEDGEMENT** .................................................................................................... III

**ACADEMIC CONTRIBUTION** .......................................................................................... V

**ABSTRACT** ....................................................................................................................... 1

**CHAPTER 1** ..................................................................................................................... 4

A REVIEW OF THE LITERATURE ......................................................................................... 4

**Introduction** .................................................................................................................... 4

1.1 Immune response: An overview ................................................................................. 4

1.2 Initial recognition of danger: PAMPs and DAMPs ....................................................... 7

1.3 ATP: The endogenous “signal 0” ................................................................................ 10

1.4 ATP release from cells ................................................................................................. 11

1.5 Purinergic Signalling- An overview .......................................................................... 15

1.5.1 Discovery of Purinergic Signalling ....................................................................... 15

1.5.2 Classification and distribution of purinergic receptors .......................................... 15

1.5.3 Purinergic receptors in immune cells .................................................................... 19

1.5.4 Role of ATP in short-term and long term (trophic) Purinergic Signalling ............... 21

1.5.5 P2X receptors in macrophages and microglia ......................................................... 22

1.5.6 P2X7 receptor structure ......................................................................................... 24

1.5.7 Membrane pore formation by P2X7 receptors ....................................................... 27

1.6 P2X7R in inflammation ............................................................................................... 29

1.7 Pharmacological and therapeutic roles of P2X7 ......................................................... 31

1.8 Overall Aims ............................................................................................................... 35

**CHAPTER 2** ..................................................................................................................... 36
MATERIALS AND METHODS........................................................................................................36

Materials ..................................................................................................................................36
2.1 Drugs and Reagents .......................................................................................................36
2.2 Chemicals.........................................................................................................................37

Methods ..................................................................................................................................37
2.3 Cell culture .......................................................................................................................37
2.4 Transfections ...................................................................................................................38
2.5 Dye uptake experiments .................................................................................................39
2.6 Measurement of Ca\textsuperscript{2+} concentration using Fura-2 AM.............................40
2.7 Immunostaining and flow cytometric analysis ................................................................40
2.8 Enzyme-Linked Immunosorbent Assay (ELISA) ............................................................41
2.9 MTS cell viability assay ...................................................................................................43
2.10 Measurement of caspase 3/7 activity using ImageXpress ..............................................45
2.11 Measurement of current amplitudes using IonFlux-16 system .......................................45
2.12 Cellular reactive oxygen species (ROS) measurement using CM-H\textsubscript{2}DCFDA .......47
2.13 Mitochondrial superoxide measurement using MitoSOX\textsuperscript{TM} Red ..................48
2.14 Rat macrophage isolation .............................................................................................49
2.15 Statistical analysis .........................................................................................................49

CHAPTER 3 .................................................................................................................................51

EFFECT OF TETRACYCLINE DERIVATIVES ON PURINERGIC RECEPTORS ..........51

3.1 Introduction .....................................................................................................................51
Minocycline: an anti-inflammatory and neuroprotective tetracycline antibiotic .................51
Possible molecular targets of MINO ......................................................................................54
3.2 Rationale for the research ..............................................................................................55
3.3 Research Question .........................................................................................................55
3.4 Results ..................................................................................................................................................................... 56
3.4.1 P2X7 responses in BV-2 microglia .......................................................................................................................... 56
3.4.2 P2X7-mediated pro-inflammatory responses ........................................................................................................... 61
3.4.3 Investigating the selectivity of MINO .......................................................................................................................... 64
3.4.4 Investigating internalisation of P2X7 receptors ........................................................................................................... 71
3.4.5 Investigating the effect of MINO on YOPRO-1 fluorescence ..................................................................................... 71
3.4.6 Investigating the pharmacological properties of MINO towards P2X7 and P2X4. ...................................................... 74
3.7 Investigating the effect of tetracycline and its structural analogues on P2X7 ................................................................. 77
3.8 Effect of MINO and DOX on P2X7 responses using primary macrophages ................................................................. 80
3.4.9 Investigating the effect of MINO and DOX on P2Y1 and P2Y2 receptors ................................................................. 82
3.4.10 Investigating the effect of MINO and DOX on TRPV-1 ion channels ................................................................. 84
3.5 Key Points ..................................................................................................................................................................... 86
3.6 Discussion .................................................................................................................................................................... 86
Mechanism of action of MINO via P2X receptors ........................................................................................................... 91
3.7 Conclusion .................................................................................................................................................................. 93

CHAPTER 4 ........................................................................................................................................................................... 94
PURINERGIC RECEPTOR MODULATION BY GINSENSIDES ......................................................................................... 94
4.1 Introduction ............................................................................................................................................................... 94
4.2 Biodegradation and bioavailability of ginsenosides ...................................................................................................... 99
4.3 Mechanism of ginsenosides action ........................................................................................................................... 100
4.4 Research Question .................................................................................................................................................... 105
4.5 Results ........................................................................................................................................................................ 106
4.4.1 Effect of ginseng extract G115 on human P2X7 ...................................................................................................... 106
4.4.2 Potentiation of human P2X7 mediated dye uptake by the ginseng metabolite CK ............................................. 107
4.4.3 Potentiation of human P2X7 mediated Ca$^{2+}$ influx by ginseng metabolite CK ............................................. 107
4.4.4 Lack of difference in effect of CK among different species of P2X7 ..........108
4.4.5 Potentiation of P2X7 mediated cell death by ginseng metabolite CK ..........109
4.4.6 Investigating effect of ginsenosides on P2X4 ...........................................118
4.4.7 Effect of ginsenosides on P2X4 mediated dye uptake ......................................118
4.4.8 Ginseng metabolite CK increases Ca\(^{2+}\) influx via P2X4 activation ...............118
4.4.9 Effect of selected ginsenosides on ATP concentration response curve in HEK-P2X4 cells ........................................................................................................................................119
4.4.10 Effect of selected ginsenosides on Ca\(^{2+}\) responses in HEK-P2X4 cells ..........119
4.4.11 Effect of ginsenosides on P2X4 inward currents using high throughput IonFlux-16 automated patch clamp system .................................................................120
4.4.12 Effect of P2X4 and ginsenosides on cell viability ..........................................120
4.4.13 Role of ginsenosides in modulation of P2X2 receptor function .....................130
4.4.14 Effect of ginsenosides in modulation of P2Y1 and P2Y2-mediated Ca\(^{2+}\) responses 134
4.6 Key Points ...........................................................................................................137
4.7 Discussion ............................................................................................................137
4.8 Conclusions ........................................................................................................143

CHAPTER 5 .............................................................................................................144

SELECTED GINSENOSIDE S MODULATE P2X7 RECEPTOR RESPONSES IN MACROPHAGES .........................................................................................................................144

5.1 Introduction ........................................................................................................144
5.2 Research Question ..............................................................................................146
5.3 Results ................................................................................................................146
5.4.1 J774 macrophages express functional P2X7 ................................................146
5.4.2 CK and Rd enhance P2X7 mediated Ca\(^{2+}\) influx in J774 macrophages ..........149
5.4.3 Potentiation of P2X7 mediated YOPRO-1 uptake and Ca\(^{2+}\) influx by CK increases in a concentration dependent manner ..............................................................149
5.4.4 LPS does not affect P2X7 channel activity in J774 macrophages .................155
5.4.5 CK and Rd increase the ATP induced cell death ........................................ 159
5.4.6 CK and Rd increase P2X7 mediated cellular ROS production .................. 162
5.4.7 CK and Rd increase P2X7 mediated mitochondrial superoxide (mtSOX) production 166
5.8 CK increases caspase 3/7 activity in ATP activated J774 macrophages .......... 171
5.4.9 CK and Rd increase caspase 3/7 activity in the presence of LPS in ATP activated macrophages .................................................................................. 178
5.4.10 Effect of selected ginsenosides on P2X7 mediated responses in primary peritoneal macrophages ................................................................. 181
5.4.11 CK and Rd increase Ca2+ influx in ATP activated primary peritoneal macrophages . 181
5.4.12 Effect of ginsenosides on cell viability in primary peritoneal macrophages ...... 182
5.4.13 Effect of ginsenosides on mitochondrial superoxide production in primary peritoneal macrophages ........................................................................................................ 182
5.4.14 Effect of CK on caspase 3/7 activity in primary peritoneal macrophages .......... 182
5.4.15 Effect of ginsenosides on cytokine secretion in J774 macrophages .............. 189
5.4.16 Effect of ginsenosides on cytokine secretion in primary peritoneal macrophages .... 194
5.5 Key results from this study are summarised in Figure 5.30 ......................... 198
5.6 Discussion ........................................................................................................... 200
5.7 Conclusion ......................................................................................................... 205
General Conclusions ......................................................................................... 215
Future Directions ............................................................................................. 215
REFERENCE ........................................................................................................ 218
APPENDICES ...................................................................................................... 267
Cell Line Information ......................................................................................... 267
A. HEK-293 cells .................................................................................................. 267
B. J774A.1 macrophage cell line ......................................................................... 268
C. BV-2 microglia cell line .................................................................................. 269
D. Buffers ......................................................................................................................... 270

Phosphate Buffer Saline (PBS, pH 7.3) ............................................................................. 270
Electrolyte Low Divalent Buffer (E\textsubscript{LDV}, pH 7.3) ................................................. 270
Physiological buffer (E\textsubscript{total}, pH 7.3) ..................................................................... 271
Krebs-HEPES buffer (pH 7.3) ........................................................................................... 271
HBSS-HEPES Buffer (pH 7.3) ........................................................................................... 272
Ammonium chloride lyse (10X concentration) ................................................................. 272
LIST OF FIGURES

Figure 1.1: Interaction of innate and adaptive immune responses (11)..........................6
Figure 1.2 PAMPs and DAMPs : Critical mediators of immune system .........................9
Figure 1.3 Sources of ATP and its effects on immune system .......................................12
Figure 1.4 Extracellular ATP acts as a legend for Purinergic Signalling ........................14
Figure 1.5 Classification of purinergic receptors (71)..................................................17
Figure 1.6: Extracellular Purinergic Signalling ............................................................20
Figure 1.7: General structure of P2X7 ........................................................................26
Figure 1.8 Domain structure of P2X7 ........................................................................26
Figure 2.1 Sandwich ELISA protocol (243) ..................................................................43
Figure 2.2 Schematic representation of the MTS cell viability detection method ..........44
Figure 2.3 The special FluxIon plates used for automated whole cell current measurement and the pattern within each plate .................................................................46
Figure 3.1 Chemical structure of MINO (C_{23}H_{27}N_{3}O_{7}) .......................................51
Figure 3.2 A schematic diagram displaying P2X7 and associated signalling pathways ......53
Figure 3.3 Effect of MINO on P2X7 mediated YOPRO-1 uptake in BV-2 microglial cells .57
Figure 3.4 Effect of MINO on P2X7 activated by various concentrations of ATP in BV-2 microglial cells ........................................................................................................58
Figure 3.5 Effect of MINO on P2X7 mediated Ca^{2+} influx in BV-2 microglial cells ....59
Figure 3.6 Dose dependent inhibitory effect of MINO on P2X7 mediated Ca^{2+} influx in BV-2 microglial cells ........................................................................................................60
Figure 3.7 Effect of MINO on IL-1β release in BV-2 microglial cells as measured by ELISA .........................................................................................................................62
Figure 3.8 Effect of MINO on cell proliferation in BV-2 microglial cells as measured by Cell Titer 96® AQueous One Solution Assay .................................................................63
Figure 3.9 Effect of MINO on YOPRO-1 uptake in HEK-293 cells expressing rat P2X7 .....66
Figure 3.10 Effect of MINO on YOPRO-1 uptake in HEK-293 cells expressing rat P2X4 ...67
Figure 3.11 Effect of MINO on ATP-induced dye uptake in HEK-293 cells stably expressing human P2X7 ........................................................................................................68
Figure 3.12 Effect of MINO on ATP-induced dye uptake in HEK-293 cells stably expressing human P2X4 ........................................................................................................69
Figure 3.13 Comparison of the effect of MINO on ATP-induced dye uptake in HEK-293 cells stably expressing human P2X7 or human P2X4. ......................................................70
MINO does not quench fluorescence of YOPRO-1 dye and does not alter receptor expression on the cell surface.

MINO does not reduce the maximum YOPRO-1 uptake in lysed cells.

MINO acts as a non-competitive antagonist at human P2X7.

MINO acts as a non-competitive antagonist at human P2X4 receptors.

Effect of tetracycline class antibiotics on ATP-induced dye uptake in HEK-P2X7.

TET analogue DOX also attenuates P2X7 mediated responses.

MINO and DOX have an inhibitory effect on P2X7 mediated Ca^{2+} influx in primary peritoneal macrophages.

MINO inhibits P2Y1 and P2Y2 mediated intracellular Ca^{2+} influx in HEK-293 cells.

MINO inhibits dye uptake in T-Rex-HEK rat TRPV-1 cells.

Chinese herb *Panax ginseng* has a number of reported medicinal applications.

Possible biotransformation of ginsenosides by lactic acid bacteria in human intestines.

Standardised ginseng extract G115 increases P2X7 mediated YOPRO-1 uptake.

Some protopanaxadiol ginseng compounds increase P2X7-mediated YOPRO-1 uptake.

CK acts as a positive allosteric modulator of human P2X7 receptors.

CK acts as a positive allosteric modulator of human P2X7 receptors activated by BzATP.

Rd enhances the sustained dye uptake associated with hP2X7 receptor activation.

CK enhances the sustained Ca^{2+} response associated with hP2X7 receptor activation.

There is no difference in the potentiating effect of selected ginsenosides on P2X7 mediated YOPRO-1 uptake in humans and rats.

CK enhanced the ability of ATP to cause cell death in HEK-P2X7 cells.

Effect of ginseng extract G115 on ATP-induced dye uptake in HEK-293 cells expressing human P2X4.

Effect of ginsenosides on ATP-induced dye uptake in HEK-293 cells expressing human P2X4.
Figure 4.13 ATP-induced dye uptake in HEK-293 cells expressing human P2X4 in the presence of varying doses of CK .................................................. 124
Figure 4.14 Concentration dependent effect of ginsenosides on ATP-induced dye uptake in HEK- human P2X4 cells ................................................................. 125
Figure 4.15 CK enhances the sustained Ca\(^{2+}\) response associated with hP2X4 receptor activation. ................................................................. 126
Figure 4.16 CK acts a positive modulator of P2X4 receptor .................................................. 127
Figure 4.17 Effect of ginsenosides on current amplitude in HEK-hP2X4 cells ....................... 128
Figure 4.18 ATP does not induce cell death in HEK-hP2X4 cells ........................................... 129
Figure 4.19 Effect of ginsenosides on P2X2a dye uptakes in transiently transfected HEK-293 cells ............................................................................................... 131
Figure 4.20 Effect of ginsenosides on P2X2a Ca\(^{2+}\) influx in transiently transfected HEK-293 cells ............................................................................................... 132
Figure 4.21 Selected ginsenosides do not alter P2Y mediated Ca\(^{2+}\) influx .......................... 135
Figure 4.22 Comparative analyses of ginsenosides on P2X7, P2X4 and P2X2a mediated YOPRO-1 uptake and Ca\(^{2+}\) responses .................................................. 136
Figure 5.1 Expression of functional P2X7 receptor in J774 macrophages ............................... 148
Figure 5.2 Selected ginsenosides enhance the sustained Ca\(^{2+}\) response associated with P2X7 receptor activation in J774 macrophages ............................... 151
Figure 5.3 CK increases P2X7 mediated dye uptake in J774 macrophages in a concentration dependent manner ................................................................. 152
Figure 5.4 Effect of CK on Ca\(^{2+}\) influx in ATP activated P2X7 responses in J774 macrophages ............................................................................................... 153
Figure 5.5 Effect of CK on ATP-induced Ca\(^{2+}\) influx in P2X7 responses in J774 macrophages ............................................................................................... 154
Figure 5.7 Effect of selected ginsenosides on Ca\(^{2+}\) influx in LPS primed J774 macrophages ............................................................................................... 158
Figure 5.8 Effect of LPS priming on cell viability in ATP stimulated J774 macrophages in the presence of ginsenosides ........................................................................ 161
Figure 5.9 Optimisation of CM-H\(_2\)DCFDA assay with H\(_2\)O\(_2\) as positive control in the presence and absence of LPS priming in J774 macrophages for measurement of cellular ROS ........................................................................ 163
Figure 5.10 Effect of selected ginsenosides on cellular ROS production in the presence and absence of LPS priming in J774 macrophages in CM-H\(_2\)DCFDA assay .......... 164
Figure 5.11 Effect of ginsenosides on cellular ROS production in the presence and absence of LPS priming in J774 macrophages. ................................................................. 165
Figure 5.12 Effect of ginsenosides on mitochondrial superoxide (mtSOX) productions in the presence of FCCP and Actinomycin in ATP activated J774 macrophages .......... 168
Figure 5.13 Effect of ginsenosides on mitochondrial superoxide productions in ATP activated J774 macrophages ................................................................. 169
Figure 5.14 Effect of ginsenosides on mitochondrial superoxide production in presence of 100 ng/ml LPS in ATP activated J774 macrophages ......................................... 170
Figure 5.15 Measurement of caspase 3/7 activity in the presence of apoptotic inducer staurosporine for optimisation of assay ..................................................... 174
Figure 5.16 NucView 488 3/7 caspase activity in presence of 0.01% DMSO, 0.5 ¿M STR and 20 ¿M CK ................................................................................................. 175
Figure 5.17 Measurement of caspase 3/7 activity in the presence of selected ginsenosides CK, Rd, Rb1 and Rh2 ........................................................................... 176
Figure 5.18 NucView 488 3/7 caspase activity in presence of 500 ¿M ATP, ATP + CK and ATP + CK + AZ10606120 ............................................................................ 177
Figure 5.19 Measurement of caspase 3/7 activity in the presence of selected ginsenosides CK, Rd, Rb1 and Rh2 in LPS primed J774 macrophages. ................................. 179
5.10 Effect of selected ginsenosides on P2X7 mediated responses in primary peritoneal macrophages ...................................................................................... 181
Figure 5.21 Effect of ginsenosides on Ca²⁺ influx in primary peritoneal macrophages ...... 184
Figure 5.22 Effect of CK on ATP-induced Ca²⁺ influx in P2X7 responses in primary peritoneal macrophages .................................................................................. 185
Figure 5.24 Effect of ginsenosides on mtSOX production in primary peritoneal macrophages ................................................................................................. 187
Figure 5.25 TNF-α production by J774 macrophages with and without LPS stimulation .... 191
Figure 5.26 IL-1β productions by J774 macrophages with and without LPS stimulation ... 192
Figure 5.27 IL-6 production by J774 macrophages with and without LPS stimulation ...... 193
Figure 5.28 TNF-α production by primary peritoneal macrophages ± LPS stimulation ...... 195
Figure 5.29 IL-1β production by primary peritoneal macrophages ± LPS stimulation ........ 196
Figure 5.30 IL-6 production by rat macrophages with and without LPS stimulation ........ 197
Figure 5.30 Diagrammatic representations of the experimental findings in the present study. ........................................................................................................... 198
## LIST OF TABLES

Table 4.1 Classification of ginsenosides based upon their backbone structures [adapted from Chen et al. 2009 (368)] ................................................. 97

Table 4.2: Substituent R groups of some of the common ginsenosides [adapted from Sergiy Oliynk and Seikwan oh, 2013 (369)] ................................................. 98

Table 4.3 Recent studies correlating the physiological effects of ginsenosides have been correlated with their capability to modulate various signalling pathways. ...................... 103

Table 4.4 Comparative list of EC$_{50}$ values of ATP for human and rat P2X7 in the presence of selected ginsenosides ................................................................. 109
<table>
<thead>
<tr>
<th>Symbols</th>
<th>Meanings</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CK</td>
<td>Compound K</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disorder</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Danger associated molecular patterns</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetate</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S Food and Drug Administration</td>
</tr>
<tr>
<td>FRT-site</td>
<td>Flippase recognition target</td>
</tr>
<tr>
<td>GSK</td>
<td>GlaxoSmithKline</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s buffer saline solution</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human embryonic kidney-293 cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IL-18</td>
<td>Interleukin-18</td>
</tr>
</tbody>
</table>
IL-1β  Interleukin-1 β  
IP₃  Inositol triphosphate  
LDH  Lactate dehydrogenase  
LPS  Lipopolysaccharide  
MAPK  Mitogen activated protein kinases  
MINO  Minocycline  
NAD⁺  Nicotinamide adenine dinucleotide  
NADPH  reduced Nicotinamide adenine dinucleotide phosphate  
NANC  Non-adrenergic Non-cholinergic  
NF-κB  Nuclear factor kappa-light-chain-enhancer of activated B-cells  
NLRP-3  Nod-like receptor protein-3  
NMDG⁺  N-methyl D-glucosamine  
NMMHC-IIA  Non-muscle myosin heavy chain IIA  
NOD  Nucleotide-binding oligomerization domain  
NSAID  Nonsteroidal anti-inflammatory drugs  
PAMPs  Pathogen associated molecular patterns  
PLC  Phospholipase C  
PRR  Pattern recognition receptors  
RBC  Red blood cells  
RNA  Ribonucleic acid  
ROS  Reactive oxygen species  
RPMI  Roswell Park Memorial Institute medium  
S.E.M  Standard error of the mean  
STAT-3  Signal transducer and activator of transcription 3  
TET  Tetracycline  
TIGE  Tigecycline  
TNF-α  Tumour necrosis factor alpha
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>TNP-ATP</td>
<td>Trinitophenol-adenosine triphosphate</td>
</tr>
<tr>
<td>TRPA-1</td>
<td>Transient receptor potential cation channel, subfamily A, Member 1</td>
</tr>
<tr>
<td>TRPV</td>
<td>Transient receptor potential cation channel subfamily V</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
</tr>
<tr>
<td>YOPRO-1</td>
<td>YO-PRO™-1Iodide</td>
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ABSTRACT

Inflammation is a biological response invoked by the body when challenged by harmful stimuli such as pathogens, injury or irritants. Inflammatory responses can be classified as being acute or chronic. In cases where the acute inflammatory response is unable to eliminate the infection or resolve the damage, the characteristics of the immune response are altered. The neutrophil response is supplanted by monocyte-derived macrophages and if there is infection, T cells from the adaptive immune system are employed as well. If the infection persists, a state of chronic inflammation ensues. This is accompanied by continued production of pro-inflammatory cytokines. The chronic inflammatory response may cause tissue destruction due to the production of reactive oxygen species (ROS) and hydrolytic enzymes. The over-production of pro-inflammatory cytokines contributes to the pathophysiology of many diseases such as heart disorders, cancer, obesity, stroke, chronic respiratory diseases and diabetes. According to the World Health Organisation (WHO), the chronic inflammatory diseases are the major cause of deaths in the world. According to the Australian Institute of Health and Welfare, 50% of Australians were reported having 1 of 8 chronic inflammatory diseases is high. The economic impact of chronic inflammatory disease is high. For example, the direct healthcare costs incurred by patients of chronic obstructive pulmonary disorders in the year 2010 were calculated to be approximately $50 million. There is a growing need to identify molecular targets (enzymes and receptors) which are key regulators of inflammatory pathways and to develop approaches to modulate them in order to attenuate inflammatory damage. Previous studies have indicated significant involvement of adenosine triphosphate in initiating and regulating inflammatory pathways.

Extracellular ATP (eATP) is involved in activating inflammatory responses and is considered as an acute ‘danger signal’ or DAMP (Danger Associated Molecular Pattern). eATP can bind to and activate purinergic receptors. Purinergic receptors, also known as purinoceptors, are a
family of plasma membrane complexes that are found in almost all mammalian tissues and are activated by purines. P2X7, a trimeric purinergic ion channel is commonly expressed on immune cells such as microglia, monocytes, macrophages and dendritic cells. P2X7 is characterised by the formation of a large non-selective membrane pore following prolonged exposure to its ligand ATP, which allows the passage of small organic molecules (up to 900 Da in size) as well as high calcium flux into the cells. P2X7 regulates the maturation and secretion of inflammatory cytokines such as IL-1β and IL-18. Alteration in function and expression of P2X7 has been implicated in the development and progression of many disease types such as neurodegenerative disorders, cancer, neuropathic and inflammatory pain and pulmonary fibrosis. Inhibition of receptor or deletion of the P2X7 gene has shown a down-regulation of inflammatory pathways. Thus, the P2X7 receptor is a potential drug target for treating inflammation-based disorders. Identification and characterisation of small molecular weight modulators for this receptor are important for establishing effective treatments for inflammation-related disorders. Several pharmaceutical companies (GSK, AstraZeneca, and Evotech) have been developing novel antagonists for the past decade and some of them have entered clinical trials for treatment of rheumatoid arthritis.

The project described in this thesis explores existing compounds that have been demonstrated to have pharmacological actions on P2X7-dependent signalling pathways and characterises the effects of two unrelated groups of compounds found to have pharmacological action on P2X7, namely, tetracycline antibiotics and ginsenosides derived from the Chinese herb ginseng.

Minocycline is a broad-spectrum antibiotic, which has been reported to have anti-inflammatory properties independent of its anti-microbial properties. Minocycline is an endogenous microglial inhibitor. In this study, it was observed that by inhibiting P2X7 responses, minocycline inhibited the influx of Ca^{2+} ions, and hence decreased pro-inflammatory cytokines
secretion, ROS generation and subsequent cell death. These results provided evidence that the anti-inflammatory action of minocycline is due, in part, to blocking the P2X7 receptor.

*Panax ginseng* is a Chinese herb which has been used traditionally for treatment of anaemia, diabetes, inflammation of the stomach lining (gastritis), fever, chronic obstructive pulmonary disease (COPD), and asthma, all of which are diseases where resolution of inflammation or immune responses are compromised. The main bioactive components among these are ginseng saponins, also known as ginseng glycosides or ginsenosides. Previous work in our laboratory has indicated that specific ginsenosides potentiate P2X7 responses. In this study, selected ginsenosides (CK, Rd, Rb1 and Rh2) were shown to act as positive allosteric regulators of P2X7 receptors in HEK-293 cells overexpressing P2X7. Potentiation of channel activity by these ginsenosides was found to translate to physiological responses mediated by P2X7 in both J774 macrophages and primary rat macrophages. These results indicate that ginsenosides may re-sensitize the immune system in individuals with sub-optimal immune responses by potentiating P2X7 responses in macrophages.

Overall, this thesis describes experiments which comprehensively characterise two unrelated classes of compounds; semi-synthetic tetracycline antibiotics and *Panax ginseng* derived ginsenosides, as effective pharmacological modulators of P2X7. Due to the involvement of P2X7 in numerous diseases, there is a mounting need to characterise physiological modulators of P2X7, which can act as tools to unravel the exact mechanism by which this receptor regulates inflammatory pathways. The information gathered with the help of these physiological tools may assist in designing safe, specific and selective drugs to target the P2X7 receptor and thereby develop novel treatments for inflammatory disorders.
CHAPTER 1

A REVIEW OF THE LITERATURE

Introduction

1.1 Immune response: An overview

All organisms possess well-developed defence mechanisms in order to survive and flourish in the living world. This host-defence mechanism is the immune system, which comprises a complex series of cells, tissues and organs that interact in order to fight off any invading pathogens and keep the body healthy. The immune system becomes increasingly sophisticated as the complexity of the organism grows (1). Humans are constantly exposed to organisms that can be swallowed, inhaled or can inhabit our skin or mucosal membranes. This exposure can manifest into disease or discomfort depending upon the level of virulence of the invading pathogens and the strength of the host’s defence system (2). The indispensable role of the immune systems is most realised when it fails. A sub-optimal immune system may lead to severe infections, tumours and immunodeficiency while an overactive defence mechanism may cause allergies and autoimmune disorders (3). In humans, the immune system can be broadly classified into two sub-systems: relatively less specific (innate) and highly specific (adaptive) immunity (4).

If any bacteria, viruses, fungi or protozoans are able to cross the physical barriers to enter the body, then these are dealt with by the innate immune system (5). The characteristic feature of the innate immune response is the recruitment of neutrophils to the site of infection or injury, which is followed by a cascade of events, termed the inflammatory response, which is meant to eliminate infectious agents and restore normality (6). If this process is imbalanced or
inappropriate it may lead to conditions such as vaculitis, inflammation of connective tissue and systemic inflammatory response syndrome (7).

The adaptive immune response is a characteristic feature of higher organisms and is specific in nature as it involves antigen recognition with the help of T and B lymphocytes (8). In comparison to innate immune response, which are fast but not specific (and can cause damage to self-cells in the process), the adaptive response is slower to develop but targets particular antigens. The adaptive immune response also has memory, which makes it able to respond quicker and stronger in cases where infection reoccurs (9).

When confronted by danger signals released at sites of infection, the body first employs the innate immune response and then the adaptive immune response in order to counter this attack (10). Most opportunistic microorganisms that are able to cross the physical barriers of the body are dealt with by neutrophils and macrophages of the innate immune response. In case the innate immune response is unable to eliminate the infection or if these initial responders do not recognise the invading pathogen, then the adaptive immune response is brought into play. The adaptive immune response engages its highly specific lymphocytes to recognise and eliminate the invading pathogens. The initial adaptive response may take up to 4-7 days to develop; therefore, the innate immune cells are vital in restricting the extent of infection until the full adaptive immune response ensues (Figure 1.1) (11).
Components of innate and adaptive immunity interact with each other to combat the attacking pathogens (bacteria/fungi/parasites and viruses) in the vertebrates. Dendritic cells (DCs) are accessory cells that act as connecting links between the innate and the adaptive immune system. Dendritic cells process the antigen and present it on their cell surface to the T cells of the immune system. They are, therefore, also called the antigen presenting cells (APCs). The antigen is presented to either $T_{\text{cytotoxic}}$ or $T_{\text{helper}}$ cells.
1.2 Initial recognition of danger: PAMPs and DAMPs

In case the danger signals are microorganisms, they are recognised by the cells of the innate immune system via the conserved molecular motifs expressed by the attacking micro-organism but not present in the host organism. These conserved molecular patterns are called pathogen associated molecular patterns (PAMPs). For example, lipopolysaccharide (LPS) in bacterial cell membranes (12), zymosan in fungal cell walls (13) and profilin in protozoan cell membranes act as PAMPs (14). Thus, PAMPs are functional components of the attacking pathogen and help the immune system in differentiating self from non-self (stranger hypothesis) (6). The receptors on immune cell surfaces that recognise and bind to PAMPs are called pattern recognition receptors (PRRs) such as Toll like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, and the mannose receptor. These are expressed on innate immune cells (15).

In addition to molecular patterns displayed on the surface of invading pathogens, the host cells can also release endogenous alarm signals in order to apprise the immune system of incoming stress, unscheduled cell death or microbial infection. These endogenous signals are called danger associated molecular patterns (DAMPs). These can be intracellular proteins such as heat shock proteins, high-mobility group protein 1 (HMGB1/amphoterin) or non-protein cellular products such as ATP (adenosine triphosphate), uric acid, heparin sulphate and DNA (16). PAMP and DAMP-mediated signalling and induction of an innate immune response usually result in resolution of infection but may also result in chronic inflammation or autoimmunity by changing various cell death and survival mechanisms.

The concept that an initial signal (signal 0) is mandatory for instigation of the innate immune response was first put forward by Charles Janeway in 1989 (17). He proposed that recognition of PAMPs and DAMPs by the innate immune system was the first step for initiation of the immune response, which was followed by a series of events involving components of both the
innate and the adaptive immune response, which work in collaboration in order to eliminate
the imposing threat by the incoming danger signal.

This concept was thoroughly reviewed in 2002 by Medzitov and Janeway (18), where they
explained that upon activation by invading pathogen (PAMPs), the cells of the innate immune
response undergo differentiation into effector cells. In case, these effector cells are able to get
rid of the infection by themselves, the adaptive immune response is not switched on. However,
if the elimination of the pathogen is not completed by the innate immune response, the adaptive
immune response is called into action. The information regarding the nature of the pathogenic
microbe is relayed to the cells of the adaptive immune response by components of the innate
immunity. This is done via the help of co-stimulatory molecules, such as CD80 and CD86
present on the surface of antigen presenting cells (APCs). Other theories to explain the immune
system activation have also been put forward. Most notable of which is Polly Matzinger’s idea
that the immune system becomes activated in response to danger signals sent out by dying or
injured cells rather than by self/non-self recognition or via Janeway’s pathogen-PRR binding.
According to her “danger model”, the immune system is consistently updated in response to
cellular damage (19). Even though Matzinger’s danger signal theory is not accepted in its
entirety by most immunologists, the term DAMP was first described by Seong and Matzinger
in 2004 and is still used to describe various immunological reactions (20). It is now considered
that in addition to the external pathogens attacking the body, the immune system can be
activated by endogenous danger signals as well. ATP is an omnipresent molecule, which is a
fundamental energy donor as well as an efficient cell-to-cell communicator (21). Besides these
roles, ATP is also an alarm signal or DAMP, which stimulates the host immune response to
protect against stress or injury. Figure 1.2 illustrates some of the signalling pathways that are
initiated by various molecules acting as PAMPs and DAMPs for the immune system (22).
Figure 1.2 PAMPs and DAMPs: Critical mediators of immune system (22)

Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern molecules (DAMPs) activate various signalling pathways. LPS activates both the TRIF-dependent TLR4 and MyD88-dependent pathways. The MyD88-dependent pathway is responsible for NF-κB and MAPK activation, which controls induction of pro-inflammatory cytokines. The TRIF-dependent pathway activates IRF3 by TBK1/IKKe, which is required for the induction of IFN-inducible genes. TLR1-TLR2 and TLR2-TLR6 recognize bacterial triacylated lipopeptide or diacyl lipopeptide, respectively, and recruit TIRAP and MyD88 at the plasma membrane to activate the MyD88-dependent pathway. TLR5 recognizes flagellin and activates the MyD88-dependent pathway. TLR3, TLR7, TLR8, and TLR9 reside in the endosome and recognize dsRNA, ssRNA, CpG DNA, or mitochondrial DNA. DAMPs such as HMGB1, S100s, and HSPs recognize the RAGE end products, TLR4 or TREM-1 and activates the MyD88-MAPK-NF-κB pathway. HMGB1 and RAGE activate the TLR9-MyD88 dependent pathway, which contributes to autoimmune pathogenesis. CD24 is a negative receptor to inhibit the DAMP-induced TLR4 pathway. ATP binding of the P2X7 receptor increases activation of caspase-1 by the NALP3 inflammasome and other nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) to promote secretion of IL-1β and IL-18.

*LPS-Lipopolysaccharide, TRIF- TIR-domain-containing adapter-inducing interferon-β, TLR4- Toll-like receptor 4, MyD88- the myeloid differentiation factor 88, MAPK-mitogen-activated protein kinase, TBK1- TANK-binding kinase 1, IFN- Interferon, TIRAP -TIR adapter protein, HMGB1-High mobility box-1, S100- S100 proteins, HSP- heat shock proteins, RAGE- receptor for advanced glycation, TREM-1-triggering receptor expressed on myeloid cells-1
1.3 ATP: The endogenous “signal 0”

ATP is an ancient and fundamentally important biological molecule involved in vital cellular processes (23). It was discovered independently by Karl Lohmann in Germany and by Cyrus Hartwell Fiske and Yellagapradha SubbaRow in the USA in the year 1929 (24). Due to its ubiquitous presence and its role as an energy donor in most metabolic reactions, ATP is considered as the most important intracellular biochemical in living systems. The fact that ATP and its metabolite adenosine may have an extracellular role in the heart and coronary blood vessels was first demonstrated by Drury and Szent-Györgyi in 1929 (25). A number of studies then focused on the action of ATP on heart muscles and its subsequent effect on cardiovascular functions (26, 27). The effect of ATP in the nervous system was also studied by injecting ATP into various parts of the cat brain where a number of electrophysiological and biochemical changes were observed (28).

One of the first pieces of evidence that ATP may have a neurotransmitter role came from studies conducted by Holton et al. where it was reported that antidromic stimulation of the great auricular nerve of a rabbit ear artery caused ATP release, indicating some role of ATP in vasodilation (29). Buchthal and Folkow also observed that acetylcholine-mediated contraction of skeletal muscle fibres in frogs was enhanced in the presence of ATP (30). Studies conducted on the physiological and pharmacological role of adenosine and its derivatives indicated a strong extracellular role of these purines. In 1963, Bernes proposed that adenosine was the compound responsible for the causing vasodilation in myocardial hypoxia (31). This hypothesis was later replaced by Geoffrey Burnstock’s hypothesis that stress and hypoxia lead to release of ATP from endothelial cells which acts upon certain receptors present in these cells, causing coronary vasodilation associated with myocardial hypoxia (32). The accumulating evidence from these pioneering studies conducted on adenylyl compounds quashed any initial resistance to the idea of ATP’s extracellular role.
It is known that the cellular concentration of ATP varies from 3-10 mM while the extracellular concentration of ATP is very low (~10 nM) (33). Low extracellular concentrations of ATP are maintained by ectoenzymes that convert any Replaced by ATP released from cells to adenosine diphosphate (ADP). This ensures that in extracellular compartments the nucleotide signalling is relatively short-lived (34).

1.4 ATP release from cells

ATP is released from many cell types such as endothelial cells, astrocytes, macrophages and urothelial cells in response to mechanical disturbances such as sheer stress, hypoxia and pathogen invasion and also during necrosis (a type of cell death) (35-37). The concentration of extracellular ATP has been observed to increase by more than 3 fold in inflammatory diseases (38) such as atherosclerosis (39) and preeclampsia (40). ATP is released into the extracellular matrix by a variety of mechanisms including vesicles, secretory granules and membrane channels such as pannexins and connexin hemi-channels (41). Pannexins are transmembrane proteins that connect the intracellular matrix with the extracellular environment and have been implicated in the release of ATP and other nucleotides from cells (42, 43). Connexins are gap junction proteins which, in addition to connecting with other connexins in adjacent cells, can also exist independently within an individual cell as a hemi-channel or conduit (44). It has been shown that the connexins are involved in the release of ATP from the cell by over-expression in connexin-deficient glioma cells (45, 46). ATP is released during synaptic transmissions and platelet aggregation (47, 48). Moreover, ATP release has been reported from dying cancer cells via the Golgi apparatus/endoplasmic reticulum related pathway (49). Figure 1.3 illustrates the various stimuli which initiate the release of ATP in different cell type leading to unique cellular responses in each cell type.
ATP is released by platelets, neurons, epithelial and endothelial cells in response to various stimuli such as soluble mediators, mechanical stress or membrane depolarization. T cells, macrophages and microglia have also been suggested to release ATP following activation with antigen or bacterial lipopolysaccharide. ATP in the extracellular environment activates P2 receptors and stimulates many important responses in immunity and inflammation. From this information, it was suggested that ATP might be a short-range immune cell modulator.
The released ATP is converted to ADP and adenosine by the membrane bound enzymes called ectonucleotidases. These can be classified into four main groups which include ectonucleoside triphosphate diphosphohydrolases (CD39), ecto-5’-nucleotidases (CD73), ectonucleotide pyrophosphatase/phosphodiesterases and alkaline phosphatases (51). The extracellular ATP and its major breakdown product adenosine, can affect almost all types of immune cells by acting on a family of cell surface proteins called the purinergic receptors (52).

The ATP/adenosine balance is oppositely regulated in inflammatory responses (53). An increase in ATP release is essential for TLR mediated activation of monocytes/macrophages, inflammasome activation, cytokine release and host immune response during infection (54). The ATP concentration is increased during the initiation of inflammatory responses and the ATP concentrations are maintained during inflammation by inhibition of ATP breakdown enzymes (CD39 and CD72) by oxidative stress and TNF-α (55). The end of the inflammatory response is marked with a decrease in concentration of ATP and an increase in adenosine (Figure 1.4) (53). Lower ATP concentrations and high levels of adenosine inhibit the inflammatory response (56). Purinergic receptors responding to ATP and adenosine play a critical role in perceiving danger, triggering the immune responses and many pathophysiological changes in the living system. Purinergic Signalling is the focal point of this thesis and its detailed background is discussed in the following sections.
Inside a cell, mitochondria act as the powerhouse of the cell and generate ATP as the end product of oxidative phosphorylation reaction. ATP then acts as a ubiquitous energy source catalysing metabolic reactions and cellular processes. ATP as well as other nucleotides can be released actively from stressed cells. ATP released is converted into AMP or adenosine by ectonucleoside triphosphate diphosphohydrolase (CD39) and ecto-5′-nucleotidase (CD73). This enzymatic conversion of ATP to adenosine helps to maintain the optimum levels of extracellular ATP and also to abolish ATP signalling.

Figure 1.4 Extracellular ATP acts as a legend for purinergic signalling (34)
1.5 Purinergic Signalling - An overview

Purinergic receptors are defined as proteins on the surface of cells that can bind and respond to purines. The discovery, characterisation and physiological roles of these receptors are discussed in detail in the following sections.

1.5.1 Discovery of Purinergic Signalling

In the year 1972, after relentlessly working for over a decade on mechanical and electrical activities of smooth muscle cells, Geoffrey Burnstock put forward the then controversial hypothesis that ATP, the universal energy donor, also acted a neurotransmitter (57). He coined the term “Purinergic Signalling” for ATP mediated signalling as ATP is a purine molecule (58). Further work established that ATP was released from sympathetic nerves supplying smooth muscle (59). This discovery led Burnstock to challenge Dale’s principle of “one nerve - one transmitter” hypothesis by suggesting that ATP acted as a co-transmitter along with noradrenalin (60). The function of ATP as a co-transmitter was initially opposed (61) but later gained universal acceptance with many studies indicating that ATP acted as a co-transmitter in most peripheral nerves (62-66). The receptors specific for binding purines and initiating the subsequent signalling were named “purinergic receptors”. Based on the literature showing the neuromodulatory effects of purines in peripheral as well as central nervous systems, Burnstock made the first attempt at classifying purinergic receptors.

1.5.2 Classification and distribution of purinergic receptors

In 1978, Burnstock proposed that there are two separate sets of receptors for adenosine and ATP/ADP. He called the adenosine receptors P1 receptors and the ATP/ADP receptors P2 receptors (67). In 1985, the P2 receptors were further sub-classified into the P2X and P2Y receptors by Burnstock and Kennedy (68). In the beginning, this classification was based on the differences in affinities of the receptors for various agonists/ antagonists but later it was
established that there are significant structural and physiological differences between the two classes of P2 receptors (67). Consequently, the term P2X was used for ligand gated ion channels and P2Y for G-protein coupled receptors (Figure 1.5). The concept of Purinergic Signalling was widely accepted after the cloning and characterisation of the receptors involved. At present, International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification recognises eight distinct P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) receptors and seven P2X subunits. Of the seven P2X receptors, six subtypes can exist in homomeric form (P2X1–P2X5 and P2X7) (69), and at least six heteromeric channels (P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, and P2X4/6) (70). Figure 1.5 illustrates the presently accepted classification of purinergic receptors.
Purinergic receptors are classified into two major categories: P1 receptors that bind to adenosine and P2 receptors that bind to ATP/ADP. P1 receptors have further four subtypes: A1, A2A, A2B and A3. P2 receptors are either ligand gated ion channels called the P2X receptors - P2X and metabotropic G-protein coupled receptors called P2Y receptors.

Figure 1.5 Classification of purinergic receptors (71)
The P1 and P2Y receptors were found to be G-protein coupled receptors with 7 transmembrane domains connected by three extracellular (ELs) and three intracellular (IL) loops, the N terminus in the extracellular part and the C terminus in the intracellular part of the receptors (72). The P2Y receptors were further sub-classified on the basis of their sequence homology and their phylogenetic relationship. Members of the first group (P2Y1, 2, 4, 6 and 11) were found to share a sequence homology of 35-52% and contain a conserved Y-Q/K-X-X-R motif on their seventh transmembrane α-helix. The members of the second group (P2Y12, 13 and 14) were characterised by a sequence homology of 47-48% and a conserved K-E-X-X-L motif on transmembrane α-helix 7 (69). Some studies suggested that there might be a link between the structural homology and function in these groups (73-77). On the contrary, there are some studies indicating a functional similarity between P2Y receptors with lesser structural homology (78). Thus, the P2Y receptors are very diverse in the regulation of signalling pathways despite similarity in the amino acid sequences.

The P2X receptors were found to be structurally distinct with two transmembrane domains and a large extracellular hydrophilic loop. The first hydrophobic transmembrane region lies between amino acid 30-50 and the second lies between residues 330-353. The extracellular loop starts close to amino acid 52 and ends at position 329 (79). The loop largely extends out of the plasma membrane and contains the ligand-binding site. The P2X receptors are functional in their trimeric form i.e three polypeptide subunits come together to form an ion channel. Three ATP molecules can bind to trimeric P2X7 and can activate the channel so as to allow the influx of Na\(^+\) and Ca\(^{2+}\) ions and efflux of K\(^+\). Binding of ATP to P2X7 increases the intracellular Ca\(^{2+}\) levels and causes membrane depolarisation (80). There are seven known subunits of P2X receptors and P2X4 shares the maximum sequence homology with P2X7 in all the species tested. The purinoceptors were first described and characterised in neuronal cells.
but now, with the help of immunohistochemistry, it has been confirmed that these proteins are expressed in a number of non-neuronal cells of the body (71)

1.5.3 Purinergic receptors in immune cells

The interaction of nucleotides/nucleosides with their receptors can lead to either stimulation of the immune system or can develop immune tolerance, depending upon the quantity and the time of nucleotide exposure (16). Since the ectonucleotidases are involved in the hydrolysis of ATP and are highly expressed in all cells, their function is thought to be related to termination of P2X signalling (81). The extracellular purinergic signalling illustrating the role of ectonucleotidases in breakdown of ATP is shown in Figure 1.6. P2X signalling leads to the initiation of pro-inflammatory responses in the cells and the removal of ATP by its conversion to adenosines prevents these inflammatory changes. Due to this reason, P1 receptors are thought to be involved in preventing tissue injury and acute inflammation (82, 83). This protective role of P1 receptor mediated signalling is also confirmed by studies where CD39 and CD73 knockout mice were more prone to inflammatory tissue damage in comparison to control due to the lack of P1 receptors (84, 85). CD39 deletion or dysfunction has been associated with the development of inflammatory diseases such as Crohn’s disease and inflammatory bowel disease (86). The CD39/CD73 pathway also affects the CD4+ T regulatory cells (Tregs), the suppression of which can attenuate HIV infection (87). Taken together, these studies suggest that P1 signalling has an anti-inflammatory role as it prevents the ATP binding to the P2X7 receptor and thereby preventing the activation of pro-inflammatory pathways controlled by P2X7 receptors (88).
Simplified representation of Purinergic Signalling where ATP is released from the cells and gets converted to adenosine by the action of various ectonucleotidases. ATP and its breakdown products further act on purinergic receptors to modulate various downstream signals. E-NTPDases (CD39) are ectonucleosidase triphosphate diphosphohydrolase family of enzymes which covert ATP to ADP/AMP. E-NPPs are ectonucleotide pyrophosphate/phosphodiesterase family (CD73) which catalyse the conversion of ATP and ADP to AMP. The E-5′ nucleotidases convert AMP to adenosine.
1.5.4 Role of ATP in short-term and long term (trophic) Purinergic Signalling

The influence of purinergic receptors on signalling pathways can be either short term or long term (trophic) (90). The short-term Purinergic Signalling effects include neurotransmission, neuromodulation, secretion, chemoattraction, and platelet aggregation (91). The long-term Purinergic Signalling can affect processes such as embryonic development, cell proliferation and differentiation, angiogenesis, restenosis after angioplasty, atherosclerosis and glial cell activities. The effect of short-term signalling by purinergic receptors can be witnessed in blood vessels supplied by parasympathetic nerves. ATP is released as a co-transmitter along with noradrenaline by parasympathetic nerves and this ATP acts on P2X receptors to excite smooth muscles in the blood vessels leading to vasoconstriction (32). ATP release from endothelial cells during shear stress and hypoxia can activate P2Y receptors by autocrine signalling and cause the release of endothelial derived relaxing factor (EDRF) or nitric oxide, which may lead to vasodilation. Adenosine, formed by ATP breakdown, can also participate in short-term Purinergic Signalling by acting on P1 receptors in the muscles and leading to vasodilation (52, 92).

Long-term trophic signals are induced by ATP and adenosine binding to purinergic receptors coupled to signal-transducing effector molecules (Src, GTPases, phospholipase-A, phospholipase-D and adenylyl cyclase). These effector molecules lead to the generation of secondary messengers or protein kinases which can regulate transcriptional factors to control the synthesis of proteins required in long term trophic factors such as those involved in cell proliferation, migration, differentiation and apoptosis (90).

Purinergic Signalling plays a significant role in health and disease. Therapeutic approaches for treatment of pathological disorders caused by alteration in purinergic receptor functions can be achieved by development of selective agonists and antagonists for P1 and P2 receptors. Development of small molecule drugs which can be orally administered to the patients and are
able to target purinergic receptors, ecto-nucleotidases or ATP transporters could be a crucial step in discovering novel treatments for inflammatory disorders.

1.5.5 P2X receptors in macrophages and microglia

The various subtypes of purinergic receptors are expressed by a variety of immune cells like B-lymphocytes, dendritic cells, mast cells, microglia and macrophages (71). Purinergic receptors including P2X4, P2X7, P2Y1, P2Y2, P2Y4, and P2Y6 are expressed in macrophages. Macrophages are large phagocytic cells and components of the innate immune system that play an important role in immunity. They defend the living system through to their ability to engulf the invading microbes and pathogens (93). Macrophages can regulate activation and proliferation of lymphocytes and are also responsible for T and B cell activation via antigens or alleles (94). Macrophages exposed to antigens are said to be “activated” and display increased bactericidal capabilities (95).

In 1994, it was reported by Hickman et al. that human mononuclear phagocytes expressed P2Z/P2X7 like activity. In this study, an increase in P2X7 responses was observed as monocytes matured into macrophages (96). The macrophage responses to extracellular ATP were evaluated and it was observed that development of a P2X7 mediated macrospore following 15 minutes exposure to ATP increased intracellular Ca²⁺. Results from this study indicated that P2X7 mediated pro-inflammatory responses and polykaryon formation in macrophages (97). The role of P2X7 in macrophage fusion was also confirmed in a later study (98). P2X7 mediated spontaneous cell death in J774 macrophages was also observed (99). DiVirgilio et al. in 1996 reported the presence of P2X7 in microglia, mast cells and macrophages (100). The effect of P2X7/P2Z receptors on macrophage and lymphocyte function had already been indicated (93). ATP release by LPS stimulation of RAW264.7 macrophages resulted in increased nitric oxide production (101). Initially, P2X7 receptor’s ability to cause IL-1β release from macrophages and monocytes was thought to be a secondary
effect of P2X7 related cytotoxicity because it was well understood that factors causing apoptosis are among the most effective stimuli for IL-1β release (102). It was later understood that rather than being a side-effect of P2X7 cytotoxicity, IL-1β was a product of a complex signalling cascade that was orchestrated by P2X7. Solle et al. evaluated the role of P2X7 in IL-1β post translational processing and found that peritoneal macrophages from P2X7 knockout animals had an impaired cytokine signalling pathway and confirmed that the receptor directly affects the maturation and release of IL-1β (103). Cell death in LPS primed macrophages observed to occur via the activation of caspase-1 mechanism, independent of cytokine release (104). In addition to caspase-1, an essential role of Ca\(^{2+}\) concentration was found in regulating IL-1β production from macrophages (105, 106). Macrophage cell death following transient P2X7 activation was suggested to be independent of TLR-2 and 4, caspase-1 as well as Panx-1 (107). Pelegrin et al. in 2008 compared the IL-1β release from different macrophage type and suggested two distinct pathways of cytokine release: release of processed IL-1β via a caspase-1 cascade that could be selectively inhibited by blocking caspase-1 or Panx1, and a second mechanism of pro-IL-1β release (Ca\(^{2+}\)/caspase-1/Panx-1 independent pathway) that could be inhibited by glycine (108). Eventually, the P2X7-NLRP3 inflammasome was established, placing the role of P2X7 in right context (21, 109).

The involvement of P2X7 signalling in inflammatory and cell death pathways in macrophages has significance in development of various pathological conditions. Stimulation of macrophages and microglia resulted in secretion of IL-1β via ATP activated P2X7 responses, suggesting a role of P2X7 receptor in development of Alzheimer’s disease (110). P2X7 immunoreactivities were observed to be increased in activated microglia and macrophages of the spinal cord in amyotrophic lateral sclerosis and multiple sclerosis (111). Gain-of-function P2X7 mutations increased inflammatory responses in macrophages and polymorphism of the P2X7 gene was implicated in mood disorders and depression (112, 113). Activation of P2X7
responses in macrophages have also been cited to be beneficial in limiting *Toxoplasma gondii* infection by inflammasome activation and ROS production (114). P2X7 mediated cytokine release and cell death have been observed in microglia (115-117). Microglia are the resident immune cells of central nervous system that undergo activation and proliferation in response to inflammatory stimuli (118). Pharmacological characterisation of the P2X7 receptors was also performed in the NTW8 microglial cell line and it was confirmed that microglia expressed cytosolic pore forming P2X7 receptor which could be both negatively and positively modulated (119, 120). Brough et al. in 2002 determined that the presence of IL-1β was not necessary for the P2X7 mediated cell death, as the absence of the IL-1β gene did not affect the P2X7 related cytotoxicity whereas the absence of the receptor lead to the complete absence of cytokine release or cell death in LPS/ATP activated microglia (121). Association of P2X7 with inflammatory pathways has now been well established (122). Modulation of these pathways by targeting P2X7 in macrophages and microglia can have significant therapeutic potential.

### 1.5.6 P2X7 receptor structure

P2X7 receptor is a ligand gated ion channel which becomes activated on binding ATP. The human P2X7 gene is located on the long arm of chromosome 12 (12q24.31) and the resulting protein is made up of 595 amino acids (79). P2X7 has a molecular mass of 69 kDa but glycosylation in the extracellular loop increases this to 75-85kDa (123). Amongst all the members of the P2X family, P2X7 is the most divergent and bears the closest resemblance to P2X4 (79). The gene for the P2X4 receptor in humans is also located on 12q24.31 and radiation hybrid mapping indicates that these two genes are may be located less than 130 bp apart and these might have arisen by gene duplication (124). The general ectodomain structure of P2X7 is similar to that of P2X4 elucidated by Gouaux and colleagues in 2009. The crystal structure of P2X is described as resembling a dolphin rising from water (80).
P2X7 opening on binding with appropriate ligand, allows the passage of positively charged cations in a non-selective manner. P2X7 differs from the other P2X in many structural, function and pharmacological features. It is functional only in its homotrimeric form, gets activated at higher concentrations of ATP (EC$_{50} > 100$ μM), it is fully activated by 2’, 3’-(benzoyl-4-benzoyl)-ATP which is 10-30 times more potent than ATP (EC$_{50} = 7$ μM) and it is the largest P2X receptor (595 amino acids) bearing a long carboxyl terminal tail (242 amino acids). The activation of P2X7 by ATP/BzATP is enhanced if extracellular Ca$^{2+}$ and Mg$^{2+}$ are removed (125). Repeated exposure to the agonists results inward current through P2X7 receptor (126). These properties make P2X7 unique and its effect on downstream signalling pathways very significant in health and disease.
Figure 1.7: General structure of P2X7

P2X7 receptor is a trimeric receptor, with each of the three subunits made up of two transmembrane domains. In the above Figure, 2 transmembrane domains of one of the trimeric subunits are shown. Each subunit has an extracellular domain, which has an ATP binding site. The short N terminal and long C-terminal tail is intracellular.

Figure 1.8 Domain structure of P2X7

The figure shows different components of P2X7 and the regions encoded by them. (127).
1.5.7 Membrane pore formation by P2X7 receptors

One of the properties that set the P2X7 receptor apart from the rest of its family members is its ability to consistently form large conductance pores after prolonged exposure to its agonist ATP. For most channels, the ion selectivity remains stable after channel activation, but for a few channels including TRPV-1 (128, 129), TRPV-2 (130), TRPA-1 (131), acid-sensing ion channels (ASICs) (132) and purinergic receptors (primarily P2X7, sometimes P2X4 and P2X2), there is a dilation of the ion conductance pore on prolonged exposure to ligand (133, 134).

It is thought that binding of the ATP to P2X7 receptor for a prolonged period of time triggers the movement of TM2 that opens up a secondary permeability pathway also called the membrane pore (macropore), which allows the passage of molecules as large as 900 Da into the cell (133). The ability of P2X7 to allow otherwise impermeable organic dyes, to enter the intracellular environment, after lengthy exposure to its ligand is often employed as a reliable tool for the measurement of pore formation. Fluorescent dyes such as lucifer yellow, ethidium and YOPRO-1 iodide are used to observe the opening of the P2X7 macropore (135). Overexpression of P2X7 allows the uptake of large cationic dyes following activation by ATP (135, 136). Macrophages derived from P2X7 knockout mice are unable to exhibit this dye uptake, response indicating a lack of membrane pore formation in the absence of P2X7 (103).

The mechanism of formation of this macropore is highly debated (137-139). It has been suggested that the conversion from the channel to the pore state in P2X7 may be due to either a change in conformation within the receptor's internal selectivity filter or the sequential oligomerization of additional trimeric P2X7 subunits (140). It is understood that the TM2 domain of P2X7 is essential for the surface expression of this receptor and for pore formation. Specific amino acids in the TM2 domain necessary for pore formation have been identified (141). Some investigators argue that it is the inherent property of the ion channel while others suggest the involvement of at least one other protein. Initial studies indicated the involvement
of pannexin-1 (Panx-1), a membrane hemichannel protein, in the formation of a membrane pore on interaction with ligand bound P2X7 receptor. These studies suggest that P2X7 is physically associated with Panx-1 and regulates the formation of membrane pore which can let in large sized molecules such as organic dyes ethidium and YOPRO-1 iodide as well as the concomitant release of IL-1β from activated macrophages (108, 142, 143). Thymocytes lacking Panx-1 but containing functional P2X7 were found to show defective dye uptake during early apoptosis (144). However, later studies provided evidence against the Panx-1 being the obligatory protein involved in P2X7 pore pathway. For example, mouse macrophages deficient in the Panx-1 gene demonstrated normal ATP-mediated YOPRO-1 iodide uptake (145); siRNA knockdown of Panx-1 gene did not alter the dye uptake by mouse macrophages (146) and blockade of this hemichannel by antagonists had no effect on the pore forming activity of the ligand bound receptor (147). A study indicated that P2X7 initiated autocrine release of ATP increased the fast motility of dendritic cells and this was further enhanced by Panx-1 channels. Functional Panx-1 channels were required for migration of dendritic cells to lymph nodes even though the overall maturity of these cells was independent of Panx-1 hemichannels (148). Therefore, it is inconclusive whether Panx-1 is essential for membrane pore formation following P2X7 activation, but this protein does play a role in inflammation and immune responses in collaboration with P2X7.

There have also been some studies indicating the contribution of long C-terminal tail of P2X7 in pore formation as truncation of this tail and the presence of SNPs in this region have been observed to cause loss of pore formation by the receptor (141, 149, 150). 95% of the C-terminal end of P2X7 has been found to be required for dye uptake/membrane pore formation (151). P2X7 was reported to interact with non-muscle myosin (NMMHC-IIA and myosin Va) and extracellular ATP caused the dissociation of the P2X7-myosin complex. The dissociation of P2X7 from non-muscle myosin was suggested to be essential for the formation of a membrane pore.
pore (152). The transition to the secondary permeability pore following P2X7 activation is also studied using electrophysiology to measure the permeability of cells towards the large cation NMDG+. It is suggested that the characteristic shift in equilibrium potential observed after prolonged activation of P2X receptors is not due to pore dilation but an outcome of time-dependent change in ion concentration in the cells (153). The authors of this study suggested due to the electrophysiology techniques applied to evaluate the phenomenon and the pore dilation was only “apparent” (153). In 2016, Karasawa and Kawate generated a x-ray crystal structure of panda (Ailuropoda melanoleuca) (154). It has been suggested that membrane pore formation depends upon the lipid composition of the plasma membrane (155). Information regarding P2X7 mediated membrane pore formation may not be conclusive so far, but there is a definite need for a unifying central dogma to gain better understanding of the working and associated effects of this receptor.

The physiological relevance of this secondary permeation pathway in normal cell is often speculated (156, 157). The formation of this secondary pore in a normal cell may aid in uptake of ATP or other molecules required for meeting an enhanced metabolic demand during stress or injury, or it may be a mechanism for ATP release to drive further Purinergic Signalling. It could also be pathway for secreting chemical transmitter molecules such as glutamate (158).

1.6 P2X7R in inflammation

ATP binding to P2X7 leads to the activation of a number of downstream signalling events that may play crucial roles in the development of many disease phenotypes such as multiple sclerosis (159, 160, 161), ALS (162), Alzheimer’s disease (163), Huntington’s disease (164), cancer (165-168), neuropathic and inflammatory pain (122, 169), pulmonary fibrosis (170), ischemia (171-173) and Sjogren’s syndrome (174, 175). Single nucleotide polymorphisms (SNPs) in the P2X7 gene have been associated with alteration of receptor function which makes the individuals carrying these SNPs potentially more susceptible to certain disorders such as
systemic lupus erythematosus (176), osteoporosis (177-179), rheumatoid arthritis (180), tuberculosis (181) and inflammatory disorders (113, 182).

The association of P2X7 receptors with diseases may be due to the initiation of many signalling pathways by P2X7 activation. These include the activation of NLRP-3 inflammasome pathways (122, 183), cytokine secretion (165, 184-186), reactive oxygen species formation (187) and formation of phagolysosomes (158). The NLRP3 inflammasome is the name given (Nod like receptor family members) regulate the maturation of IL-1β (188). Activation of the NLRP3 inflammasome is associated with many pathological conditions such as brain injury, encephalitis, skin allograft rejections (21). Activation of inflammasome occurs in two steps; the priming step involves the increase in expression of inflammasome constituents such as pro-IL-1β and NLRP3 (189) and the second step involves the assembly of inflammasome components and secretion of IL-1β and IL-18 (190). The activation of caspase-1 has been linked to the efflux of K+ through P2X7 (191). Due to the association of NLRP3 inflammasome with cytokine secretion, P2X7 is considered a major physiological regulator of pro-inflammatory responses in vivo.

The significance of P2X7 in inflammatory responses is evident from the studies where absence or inhibition of the receptor has led to the downregulation of pro-inflammatory responses in the host organism. For example, genetic ablation of P2X7 decreased pathophysiological parameters in mouse model of Duchenne muscular dystrophy (192, 193). P2X7 affects graft-versus host disease and allograft rejection (194, 195). A decrease in the clinical and histopathological symptoms related to experimental autoimmune uveitis was seen P2X7 deficient mice (196). Similarly, P2X7 reduced the expansion of T_reg cells, thereby decreasing the renal injury associated with ischemia-reperfusion injury. P2X7 was implicated in the spread of HIV infection as autocrine ATP release causes activation of macrophage P2X7 causing release of microvesicles containing the HIV-1 virus (197). These studies provide strong
evidence that P2X7 is a key player in the immune responses in the body and modulation of this receptor may have significant physiological implications.

The ATP mediated activation of the P2X7 receptor is also involved in generation of reactive oxygen species in a variety of cells including microglia, macrophages, submandibular cells and erythroid cells (198). This was found to activate the NADPH oxidase 2 enzyme in these cell types (114, 199). Other signalling pathways affected by P2X7 activation include protease activation and release, prostaglandin secretion, glutamate release, cell proliferation and phagocytosis (158). All these signalling pathways are involved in inflammation. Different cell types respond differentially due to uniqueness of the effector molecules in each of these cells. Thus, there is a mounting need to characterise physiological modulators of P2X7, which can act as tools to unravel the exact mechanism by which this receptor regulates inflammatory pathways. The information gathered with the help of these physiological tools may be helpful in future for designing safe, specific and selective drugs to target the P2X7 receptor and thereby develop cures for inflammatory disorders.

1.7 Pharmacological and therapeutic roles of P2X7

The role of P2X7 in inflammation is well defined and almost all immune cells express this receptor. Therefore, the idea of regulating the activity of P2X7 by suitable drugs and modulators holds a great therapeutic potential. Inhibition of this receptor can lead to suppression of inflammatory responses which may be beneficial in ameliorating neuropathic pain and cancer to name a few. Several attempts have been made to identify, characterise or synthesise specific antagonists for P2X7. The antagonists for this receptor may be either orthosteric (binding within the ATP binding site) or allosteric (binding at a site other than the ATP binding site).
Orthosteric or competitive antagonists characterised for P2X7 include suramin or suramin-like compounds, ATP derivatives (TNP-ATP), cynoguinidine derivatives (A740003 and A804598), tetrazole derivatives (A839977, A438079) (200, 201). Of the entire orthosteric antagonist characterised for P2X7, the cyonguinidine derivatives are the most potent and selective (202). Honore et al characterised A740003 as a competitive, selective P2X7 antagonist and observed a dose dependent decrease in neuropathic pain in rats (203). Pharmacological inhibition of P2X7 by A804598 resulted in inhibition of autophagic death in muscle cells in a mouse model of Duchenne muscular dystrophy (204). Both cynoguanidine and tetrazole derivatives have antagonistic affects at a very low micromolar IC$_{50}$ value (205). ATP derivatives (oATP and TNP) on the other hand are irreversible, less selective and antagonistic at high concentrations (206).

Allosteric and non-competitive inhibitors for P2X7 responses include Brilliant Blue G (BBG) (207), KN-62 (208), AZD9056 (209), GW791343 (210, 211), GSK314181A (212), GSK1482160 (213), CE-224535(214), AFC-5128 (215), AZ-11645373 (216), AZ-10606120 (217) and JNJ-479655 (215, 218). Recently, 1, 4-Naphthoquinones compounds (A01 and A04) were tested for their inhibitory activity for P2X7 (219). BBG has been found to diminish HIV-gp120 related neuropathic pain by blocking P2X7 (220). P2X7 blockade by BBG also prevents intrauterine inflammation related pre-term birth and perinatal brain injury in mice (221). BBG prevents neuroinflammation, endocrinal dysregulation and other inflammatory disorders in which P2X7 is implicated (220, 222-225). Stokes et al. characterised the thiazolidinedione derivative AZ-11645373 in 2006 and found that while BBG was more potent in inhibiting rat P2X7 responses, human P2X7 was better blocked by AZ11645373 (226). Physiological studies conducted using this inhibitor indicate that AZ11645373 is a potent and selective blocker of P2X7 mediated inflammatory responses (227-229). Studies involving characterisation of the benzamidine compound GSK314181A for its role in inhibiting P2X7 responses have shown that
this compound can ameliorate the P2X7 mediated neuropathic pain and inflammation (212). More recently, a first in-human study was conducted for evaluating the pharmacokinetics, pharmacodynamics, safety and tolerability of JNJ-479655, a brain permeable P2X7 antagonist and it was found that this antagonist was able to downregulate LPS-induced, P2X7-mediated cytokine secretion in peripheral blood via passive penetration into the brain (230). Biotechnology company Evotec has confirmed a good safety profile of the compound EVT-401, an oral antagonist of P2X7 after successful completion of the Phase-1 study (231). Many other pharmaceutical groups including Affectis Pharmaceuticals AG, AstraZeneca, Merck, Abbott and Pfizer have characterised and patented other P2X7 inhibitors but the functional properties of these compounds are unpublished yet (232). Even though a great number of these antagonists are potent and non-competitive, still many of them can also affect other purinergic receptors such as P2X4, P2X2 and P2Ys (233). It is evident that pharmacological inhibition of P2X7 is effective in decreasing the inflammatory responses elicited by the activation of P2X7. Therefore, the pursuit to develop potent, selective and safer antagonists for P2X7 is still on-going.

In contrast, there are not many specific agonists of P2X7. Even ATP, the endogenous activator of P2X7 can stimulate the receptor only at high concentrations (EC₅₀ ≥ 100µM) (134, 234). ATP is also easily hydrolysed by ectonucleotidases, therefore the concentration of extracellular ATP is reduced down to nanomolar or low micromolar concentrations, which are more suitable concentrations for activating other purinergic receptors such as P2Y (235). Due to this reason, non-hydrolysable ATP analogues such as ATPɤS are better suited for experimental evaluation of P2X7 pharmacology, although ATPɤS is not a very potent activator of P2X7 (236). BzATP, the benzoyl benzoyl derivative of ATP, is 10-30 times more potent than ATP and also offers better selectivity for human P2X7 in comparison to other purinergic receptors (136). BzATP does get metabolised to other adenine derivatives and also has an effect on other purinergic
receptors including P2X1, P2X2, P2X3 and P2X4. There are other non-nucleotide compounds that can act as positive allosteric modulators of P2X7 (237). Studies have shown that cathelicidin or LL-37 (the antimicrobial polypeptide found in macrophages), is an activator of P2X7 receptors and has been found to induce IL-1β maturation and release (238). P2X7 potentiation by LL-37 inhibits macrophage pyroptosis and improves the survival of polybacterial septic mice (239). It has been found that P2X7 receptors are critical in mediating Aβ-mediated chemokine release, in particular CCL3, which is associated with recruitment of CD8+ T cells (163, 240). Cytoplasmic factors such as Alu-RNA accumulation in the cytoplasm have also been suggested to activate P2X7 activation independent of ATP release (241). A number of studies have provided evidence that activation of P2X7 by nucleotide as well as non-nucleotide agonists can have anti-viral, anti-parasitic and anti-inflammatory effects.

From the above analysis of literature, it can be concluded that P2X7 and its related purinergic receptors play an important role in inflammatory responses. Therefore, modulation of P2X7 receptors via appropriate pharmacological modulators may provide an effective therapeutic mechanism for modifying downstream inflammatory responses. The current thesis has therefore investigated unrelated compounds; minocycline and protopanaxadiol ginsenosides, for their potential to target and modulate P2X7 receptors.
1.8 Overall Aims

1. To characterise the inhibitory action of tetracycline antibiotics, specifically minocycline on P2X7.

2. To investigate selectivity of tetracyclines on other purinergic receptors (P2X4 and P2Ys).

3. To investigate the effect of ginsenoside formulation G115 and purified ginsenoside compounds Rb1, Rh2, Rd and compound K on purinergic receptor responses.

4. To investigate the effect of selected ginsenosides on P2X7 mediated physiological responses in the J774 macrophage cell line and primary peritoneal macrophages.

Aims 1 and 2: Investigation and characterisation of P2X7 as a molecular target of minocycline have been addressed in Chapter 3.

Aim 3: The effect of ginsenosides on P2X receptors has been discussed in Chapter 4.

Aim 4: The effect of selected ginsenosides on physiological responses mediated by P2X7 receptor in macrophages has been addressed in Chapter 5.
CHAPTER 2

MATERIALS AND METHODS

Materials

2.1 Drugs and Reagents

Dulbecco’s Modified Eagle’s Medium (DMEM), Hank’s Buffer Saline Solution (1X), HEPES (1M), Trypsin-EDTA (0.25%, phenol red) were from Gibco™. Lipofectamine® 2000, Opti-MEM, Penicillin/Streptomycin, Geneticin® (G4118) were obtained from Life Technologies (Thermo Fisher scientific), Australia.

DMEM: F12, RPMI 1640 and Foetal Bovine Serum were obtained from Bovogen, Australia. ATP (A7699), ADP (A2754), UTP (U6875), suramin (S2671), MINO (M9511), tetracycline (T7660), DOX (D9891) and tigecycline (PZ0021) were purchased from Sigma.

AZ438079, AZ10606120 and 5-BDBD were purchased from Tocris Biosciences, UK.

YO-PRO®-1 Iodide (Y3603), Fluo-4 AM, Fura-2 AM, cell permeant (F-1201), NucBlue® Live ReadyProbes® Reagent CellEvent™ (C37605), NucView 488 Caspase-3/7 Green Detection Reagent (C10423), CM-H2DCFDA and MitoSOX™ Red were obtained from Molecular Probes™ Thermo Fisher Scientific, Australia. The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit was obtained from Promega, Australia. Ginsenosides (certified as 98 % pure) Rd, Rb1, Rh2, Compound K were obtained from Chengdu Biopurify Chemicals Ltd, China. PPD1 and Rf were obtained from Sichuan Weikeqi Biological Technology Co. Ltd., China.
2.2 Chemicals

NaCl, KCl, Na₂HPO₄, KH₂HPO₄, CaCl₂.2H₂O, MgCl₂.6 H₂O, Na₂HPO₄, KH₂PO₄, NaOH, NH₄Cl, NaHCO₃ (analytical grade) were obtained from Merck, VIC, Australia. HCl and glucose were from Sigma-Aldrich, Australia. HBSS (Hank’s Buffered saline solution) and HEPES were from Gibco™ (Fisher Scientifics) Australia.

Methods

2.3 Cell culture

Mouse microglial BV-2 cells and HEK-293 cells were maintained in DMEM: F12 media (Life Technologies) supplemented with 10% foetal bovine serum (Bovogen, Australia) and 100 U/ml penicillin plus 100 µg/ml streptomycin (Life Technologies). J774 macrophages and primary peritoneal macrophages were maintained in RPMI 1640 media supplemented with 10% foetal bovine serum (Bovogen, Australia) and 100 U/ml penicillin plus 100 µg/ml streptomycin (Life Technologies). Flp-In™ 293 T-REx cell lines were used for generation of stable rat TRPV-1 cell line that ensured homogenous expression of TRPV-1 from Flp-In™ expression vector. These cells contained a single stably integrated FRT site at a transcriptionally active genomic locus. Targeted integration of Flp-In™ expression vector ensured high-level expression of the gene of interest. The Flp-In™ T-REx™-293 cell line contained pFRT/lacZeo and pcDNA™6 / TR (from the T-REx™ System) stably integrated (https://www.thermofisher.com). Co-transfection of the Flp-In™ Cell Lines with the Flp-In™ expression vector and the Flp recombinase vector, pOG44, resulted in targeted integration of the expression vector to the same locus in every cell, ensuring homogeneous levels of gene expression. Transient transfections of HEK-293 cells were performed in 35 mm petri dishes using 0.1-1 µg of plasmid DNA and Lipofectamine 2000 as per the manufacturer’s instructions. Stable cell lines
expressing human P2X7 and human P2X4 were established by clonal dilution and kept under
selection using 400 μg/ml geneticin (Life Technologies). Cells were plated into poly-D-lysine
coated 96 well plates (Thermo Fisher Scientifics) at optimised specified cell densities and
incubated overnight before experiments.

2.4 Transfections

HEK-237 cells have been used to overexpress P2X receptors. HEK-237 cells do not express
P2X receptors endogenously (242). Thus, overexpressing the P2X receptors in HEK-237 cells
allows to study the characteristics and functions. The study of structure and function of P2X
would not be as effective if the cell line used for transfection already expressed P2X receptors
as it would be difficult to isolate the responses from P2X receptors transfected from those
endogenously expressed. The plasmids for P2X7, P2X4 and P2X2a were originally obtained
from Prof Annmarie Surprenant. The stable cell lines were generated in the lab following the
described method. Cells were seeded in 35 mm dishes and allowed to grow overnight. A
cocktail of 1 μg DNA and 3 μl Lipofectamine 2000 (Invitrogen) was prepared in Opti-MEM
and incubated at room temperature for 10 minutes. This mixture was added to the petri dish
containing cells. The cells were left in a CO₂ incubator for 37 °C and media was changed after
24 hours. The cells were returned to the incubator for another 24 hours. For transient
transfections the transfected and mock cells were plated in 96 well plates and left to grow
overnight in a CO₂ incubator (5% CO₂ levels) and used to run the assay the next day. In order
to select stably transfected colonies of cells, 800 μg/ml of G418 (Geneticin®) was added to the
cells. When grown in medium containing Geneticin® selection agent, stable colonies of cells
expressing resistance markers were generated in 2-3 weeks. The selection of transfected cells
was based upon 100 % death of mock transfected cells. To generate single cell colonies, the
cells were plated in 96 well plates using limiting dilution. This method allowed the isolation of
each individual cell that carried selection marker by plating them at very low cell densities (~1
well per well in 96 well plates) and expanding colonies from those single cells in separate wells. The cells transfected with P2X receptor were tested for their functional responses by stimulating them with ATP and measuring their dye uptake (macropore formation measured by YOPRO-1 uptake, details in section 2.5) on Flexstation III. The clones that had good functional responses were grown in T 25-flasks and cryopreserved for future use.

2.5 Dye uptake experiments

For experiments with stably or transiently transfected HEK-293 cells, cells were plated at a density of 2-2.5 x 10^4 cells per well in DMEM: F12 media. BV-2 microglia were plated at density of 5 x 10^3 cells/ well in DMEM: F12 media and the J774 macrophages were plated at a density of 2 x 10^4 cells/well in RPMI media in 96-well plates (Thermo Fisher Scientific, Scoresby, Victoria, Australia). For testing the effect of MINO on dye uptake, media was replaced with low divalent cation buffer (145 mM NaCl, 5 mM KCl, 13 mM D-glucose, 10 mM HEPES and 0.2 mM CaCl_2, pH 7.3) containing 2 µM YOPRO-1 iodide (±MINO) for 30 minutes at 37 °C before the dye uptake was measured. For experiments with ginsenosides, the cells were either pre-incubated with drugs (made up in YOPRO-1 containing low divalent cation buffer) or were co-injected with the agonist automatically using a Flexstation III microplate reader (Molecular Devices, Sunnyvale, CA, USA). The dye uptake over time was recorded with excitation at 488 nm and an emission filter at 520 nm on the Flexstation III. Basal fluorescence measurements were acquired for 40 seconds followed by automatic injection of ATP (1 mM final concentration) and the kinetic measurement of fluorescence intensity was performed for 300 seconds using Softmax Pro software. Dye uptake responses were calculated as area under the curve from 50-300 seconds (or 50-180 seconds) using normalised data.
2.6 Measurement of Ca\textsuperscript{2+} concentration using Fura-2 AM.

Cells were loaded with a 2.25 µM Fura-2 acetoxyethyl (AM), 0.001 % Pluronic solution in HBSS buffer containing zero Ca\textsuperscript{2+}. Fura-2 AM was loaded on the cells for 40 minutes at 37 °C. Loading buffer was removed and replaced with standard extracellular assay buffer (145 mM NaCl, 5 mM KCl, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 13 mM glucose, 10 mM HEPES, pH 7.3). Cells were pre-incubated with drugs for 30 minutes at 37° C before measurements started and ATP was injected automatically after 15 seconds in case of MINO experiments. In assays involving ginsenosides, the pre-incubation time was 10 minutes and in most cases the drugs were co-injected with agonist automatically using Flexstation 3. Fluorescence was measured using a 485 nm excitation.

2.7 Immunostaining and flow cytometric analysis

The cells were detached from the cell culture flask by using either a sterile scraper or Trypsin-EDTA. A cell pellet was obtained by centrifuging the cell suspension at 10,000 x g for 5 minutes. The cell count and cell viability were checked using Muse® Cell Analyzer or haemocytometer. The cell suspension (final cell count-5 x 10\textsuperscript{5}cells/tube) was added to 12 x 75 mm round bottom test tubes for staining purposes. For staining proteins on the cell surface, the cells were washed with 0.5 % BSA in PBS. The cell pellet obtained after centrifugation at 10,000 x g for 5 minutes was re-suspended in pre-determined optimal concentration of a required antibody or appropriate negative control. If the primary antibody was labelled with a fluorochrome as in case of P2X7 antibody L4-FITC, then incubation on ice for 1 hour followed by washing with BSA / PBS made the cells ready for flow cytometry. The washing step involved removing the solution contacting antibody and replacing it with 300 µl of BSA/PBS solution. The base of each test tube was flicked gently so as resuspend the cell pellet. The test
tubes were centrifuged at 10,000 x g for 5 minutes. The supernatant was removed and replaced by 300 µl of PBS solution. If the primary antibody was not pre-labelled, then the cells were washed with PBS / BSA twice and then appropriate secondary antibody was added. Cells were incubated on ice for 30 minutes, washed with PBS / BSA and then re-suspended in 300 µl of PBS for flow cytometry analysis. For staining of intracellular proteins, the BD Cytofix / Cytoperm™ kit was used. The cells were washed with 1 x BD Perm/Wash buffer to obtain a pellet. The cells were fixed by incubating on ice with 250 µl of BD Cytofix / Cytoperm™ solution. Permeabilization of fixed cells was one by washing 2 times in 1 x BD Perm/Wash buffer (e.g.1ml/wash/tube). Cells were pelleted. The fixed/permeabilized cells were thoroughly re-suspended in 50 µL of BD Perm/Wash buffer containing a pre-determined optimal concentration of a required antibody or appropriate negative control. The cells were incubated for 1 hour on ice. The cells were washed 2 times with 1 x BD Perm/Wash buffer (1 ml/wash/tubes). Next, the secondary antibody was added and incubated on ice for 30 minutes. The cells were washed twice with 1 x BD Perm / Wash buffer as previously and then resuspended in staining buffer prior to flow cytometric analysis.

2.8 Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were grown in 24 wells plates at densities of 3 x 10^5 cells/ml. Drugs were added to the media at appropriate pre-calculated concentrations. The cells were incubated with the appropriate drugs (LPS/MINO /ginsenosides) for 4 hours at 37 °C in a CO₂ incubator. After 4 hours, the plates were removed, and ATP was added to “test” wells. The plates were returned to the incubator for 30 minutes. The plates were again taken out after ATP treatment and the media was collected from each well in labelled Eppendorf tubes. The tubes are centrifuged at 252 G for 5 minutes to obtain a pellet of cells. The supernatants were collected in fresh Eppendorf tubes and stored at -80 °C till the ELISA was to be performed. The BD OptEIA was used for detection of IL-1β secretion by microglial / macrophage cells. The kit contained
coating buffer, assay diluent, wash buffer and stop solution. The ELISA plates were coated with capture antibody (1:250) at 4 °C overnight. The plate was washed three times with wash buffer. The washing of the plates was done by adding 200 μl of washing buffer in each well using a multichannel pipette. The plate was then kept on a rocker (BioNova lab rocker) at 20 G for 5 minutes. The washing solution was removed by tipping the solution out of the plate gently in a waste container. The step was repeated 3 times. After the final wash, blocking buffer (200 μl) was added to each well of the 96 well ELISA plate and incubated for 1 hour at room temperature. The plates were washed twice with washing buffer and samples and standards were added for 2 hours at room temperature. The samples and standards were aspirated, and plates were washed 5 times with washing buffer before adding 100 μl detection antibodies (1:1000) to each well. After an incubation period of one hour, the detection antibody was aspirated out and wells were washed 5 times. Diluted streptavidin-HRP (100 μl) was added to each well and incubated for 30 minutes. Washing steps were repeated 7 times before adding 100 μl substrate solutions to each well. The plates were incubated in dark at room temperature. The reaction was stopped after 30 minutes by adding stop solution. Absorbance was read at 450 nm in a Clariostar plate reader. This method is called sandwich ELISA (Figure 2.1).
Figure 2.1 Sandwich ELISA protocol

A known quantity of capture antibody is bound to the surface of the ELISA maxisorp plate. The sample with the antigen is added to the plate and is captured by the antibody on the surface of the plate. A specific antibody (called detection antibody) is added which binds to antigen-antibody complex. Due to this reason, this method is called the 'sandwich' as the antigen is sandwiched between two antibodies. The detection antibodies are generally enzyme-linked secondary antibodies. The enzymes can catalyse a fluorescent or electrochemical reaction. The absorbance or fluorescence or electrochemical signal of the plate wells determines the quantity of antigen.

2.9 MTS cell viability assay

The cell viability and cell numbers were determined by using the CellTiter 96® AQeous Non-Radioactive Cell Proliferation Assay. The CellTiter 96® AQeous Non-Radioactive Cell Proliferation Assay is a colorimetric method for determining the number of viable cells in proliferation assays. It is composed of solutions of a tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazinemethosulfate; PMS). The tetrazolium was converted into soluble formazan by mitochondrial dehydrogenases of viable cells (Figure 2.2). Thus, the absorbance measured at 490 nm was a direct measure of viability as non-viable cells do not bio-reduced this dye.
For determining the cell viability following treatment with drugs, the cell suspension containing $1 \times 10^5$ cells/ml of BV-2 microglia and $1 \times 10^6$ cells/ml of HEK-293-transfected/J774 macrophages were prepared and 50 µl of this suspension was added per well so that the final seeding density was halved. The cells were allowed to grow in a CO$_2$ incubator for 24 hours. MTS solution (20 µl) was added to media in each well 4 hours before the stipulated end point time. The plate was kept in the dark in a CO$_2$ incubator for these 4 hours. At the end of the incubation time, the absorbance was read at 490 nm in a Clariostar plate reader.

Figure 2.2 Schematic representation of the MTS cell viability detection method

The CellTiter 96® AQueous One Solution Cell Proliferation Assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The MTS tetrazolium compound is reduced by cells into a coloured formazan product which is soluble in tissue culture medium. This conversion is likely caused by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells.
2.10 Measurement of caspase 3/7 activity using ImageXpress

NucView™ 488 Caspase-3 substrate detects caspase-3 activity within individual intact cells without inhibiting caspase-3 activity. The substrate consists of a fluorogenic DNA dye and a DEVD substrate moiety specific for caspase 3. The substrate, which is both non-fluorescent and non-functional as a DNA dye, rapidly crosses cell membranes to enter the cytoplasm, where it is cleaved by caspase-3 to form a high-affinity DNA dye that stains the nucleus bright green. Thus, the NucView™ 488 caspase-3 substrate is bi-functional, allowing detection of intracellular caspase-3 activity and visualization of changes in nuclear morphology during apoptosis. NucBlue® Live ReadyProbes® Reagent is a temperature stable cell permeant nuclear counter stain that emits blue fluorescence when bound to DNA. When used in conjunction with NucView™ 488, the total number of live cells and their corresponding caspase 3/7 activity can be measured.

J774 cells in 96 well glass bottomed plates (1.5-2 x 10^4 cells/well) were loaded with NucBlue (nuclear dye) and 1µM NucView 488 (caspase 3/7) for 40-45 minutes at room temperature. Compounds were then added, and images were taken following excitation at the relevant wavelengths (x10 magnification) using the ImageExpress Micro imaging system. Two sites per well were used for the analysis (n = 500-700 cells). Experimental solution used in these assays was HBSS saline containing 1mM MgCl₂ and 2 mM CaCl₂ supplemented with 20mM HEPES, pH 7.3 with 5M NaOH.

2.11 Measurement of current amplitudes using IonFlux-16 system.

For whole cell patching, a fully integrated automated patch clamp system called the IonFlux system was used. The 96-well IonFlux plates which record currents in a 20-cell ensemble format were used. Each pattern in an IonFlux plate has 8 compound wells, 2 trap recordings filled with intracellular fluid, one inlet for cells and intracellular solution and one outlet for
waste. IonFlux-16 plates were emptied, filled with reagents and compounds and primed before preparing the cells. The HEK-293 cells stably expressing P2X7 or P2X4 were used at a cell density of 4-10 x 10⁶ cells/2 ml. The cells were grown in T-175 cm² flask and on the day of the experiment, complete media was removed, and the cells are washed with 10 ml of warm Ca²⁺ and Mg²⁺ free D-PBS. The D-PBS was then replaced by 5 ml Tryp-LE. The cells were incubated at 37 °C for 5-6 minutes. The dislodged cells were then pelleted and re-suspended in extracellular solution (4 mM KCl, 138 mM NaCl, 1mM MgCl₂, 1.8mM CaCl₂, 5.6 mM glucose, 10 mM HEPES, pH 7.45, Osm - 285-290 mOsm), so that the final cell density is 2.5 x 10⁶ cells/ml. Cell suspension (250 µl) was added to each “IN” well of the Ion-flux 16 plate. The plate was loaded on to the IonFlux Automated Patch Clamp System. The drug solutions were prepared in 2 x concentrations. ATP was applied first and the responses (current amplitude) were measured. Then the cells were washed out with running buffer for 2 minutes. ATP was then reapplied in the presence of various ginsenosides. Current amplitudes were then measured for each condition using IonFlux software. Data was expressed as fold change in current amplitude.

![Image of IonFlux plate](https://ionflux.fluxionbio.com/library-docs)

**Figure 2.3 The special FluxIon plates used for automated whole cell current measurement and the pattern within each plate.**

Each pattern in an IonFlux plate has 8 compound wells, 2 trap recordings filled with intracellular fluid, one inlet for cells and intracellular solution and one outlet for waste. (https://ionflux.fluxionbio.com/library-docs)
2.12 Cellular reactive oxygen species (ROS) measurement using CM-H$_2$DCFDA

CM-H$_2$DCFDA is a chloromethyl derivative of H$_2$DCFDA (2’, 7’-dichlorodihydrofluorescin diacetate) which is a chemically reduced form of fluorescein used as an indicator for reactive oxygen species (ROS) in cells, such as detection of reactive oxygen intermediates in neutrophils and macrophages. CM-H$_2$DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent adduct (DCF) that is trapped inside the cell, thus facilitating long-term studies. DCF is a highly fluorescent compound, which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 492–495 nm and 517–527 nm respectively.

\[ \text{DCF} \]

For determining cellular ROS production by macrophages in the presence of ginsenosides, the cells were plated in black 96 well clear bottom plate (Costar®) at a seeding density of 5 x 10$^5$ cells per ml and were allowed to grow in a CO$_2$ incubator overnight. ROS indicator CM-H$_2$DCFDA (10 mM stock in DMSO) was diluted to 10 µM (2 x concentrations) using Krebs-HEPES buffer. The dye (50 µl of 2 x concentration) was added to each well. Ginsenosides (CK, Rd, Rb1, Rh2 and PPD1), ATP and positive control H$_2$O$_2$ were all prepared in 2 x concentrations and added on the dye. This diluted the dye as well as the drugs to the appropriate working concentration. The fluorescence was read from time point zero (on addition of drugs) and the readings were taken every 15 minutes for 2 hours. The plate was placed at 37 °C in between reads.
2.13 Mitochondrial superoxide measurement using MitoSOX™ Red

MitoSOX™ Red is a mitochondrial superoxide indicator (MW = 759). The production of superoxide by mitochondria can be measured using the MitoSOX™ Red reagent as it can permeate live cells and selectively target mitochondria. It is rapidly oxidized by superoxide but not by other reactive oxygen species (ROS) and reactive nitrogen species (RNS). The oxidized product is highly fluorescent upon binding to nucleic acid. For measuring mitochondrial superoxides, 50 μg of MitoSOX™ were dissolved in 13 μl of dimethylsulfoxide (DMSO) to make a 5 mM MitoSOX™ reagent stock solution. MitoSOX™ reagent working solution was prepared by diluting the 5 mM MitoSOX™ reagent stock solution in Krebs-HEPES to make a 5 μM concentration. For measuring superoxide production by macrophage mitochondria in the presence of ginsenosides, the cells were plated in black 96 well clear bottom plates (Costar®) at a seeding density of 5 x 10⁵ cells per ml and were allowed to grow in CO₂ incubator overnight. Mitochondrial ROS indicator MitoSOX was diluted to 10 μM (2 x concentrations) using Krebs-HEPES buffer. 2 x dye (50 μl) was added to each well. Ginsenosides (CK, Rd, Rb1, Rh2 and PPD1), ATP and positive controls (Antimycin-A, FCCP) were all prepared in 2 x concentrations and added on to the dye. This diluted the dye and the drugs to the appropriate working concentration. The fluorescence was read from time point zero (on addition of drugs) and the readings taken every 15 minutes for 2 hours. The plate was placed at 37°C in between reads. The mitochondrial superoxide release was quantified as fold change in ROS release from time point zero to the last measured time point.
2.14 Rat macrophage isolation

Wistar rats (control rats from experiments conducted in A/Prof. Sarah Spencer’s laboratory) were used to collect peritoneal macrophages. The dead animal was transferred in to a sterile fume hood and the abdomen area cleaned with 70% ethanol. A lateral incision was made in the abdominal wall with sterile equipment and conditions. The abdominal cavity was washed with 10 ml warm sterile PBS and the cell suspension was collected in a 15 ml sterile falcon tube. The cell suspension was centrifuged at 10,000 x g for 10 minutes. The cell pellet was then re-suspended in 1 ml RPMI-1640 media. Cells were counted using a haemocytometer (only the large cells were counted). The cells were plated out in a specific cell number as required by each experimental set up (For example- 2.5 x 10^5 cells/well were seeded for Fura-2 AM assay). Cells from each animal were considered as a separate biological sample.

Note: If there was red blood cells (RBC) contamination, 2 ml of RBC lysis buffer was added to the cell pellet after the centrifugation step and incubated at room temperature for 10 minutes on a rocker to lyse the red blood cells. Centrifugation and washing with PBS was done to remove RBCs completely.

2.15 Statistical analysis

All graphs were plotted using GraphPad Prism versions 6 or 7 (La Jolla, USA). For concentration-response graphs, concentrations were first transformed using the in-built function \( X = \log[X]\) and plotted against response (either raw data or normalised data). Curve fitting was performed using the built-in dose – response – stimulation function using a four-parameter fit with variable slope. The equation used to fit raw data is

\[
Y = \text{Bottom} + \frac{(\text{Top-Bottom})}{(1 + 10^{\text{LogEC}_{50} - X}) \times \text{hillslope}}
\]

The equation used to fit normalised data is

\[
Y = \frac{100}{(1 + 10^{\text{LogEC}_{50} - X})}
\]
$EC_{50}$ values were defined as the concentration of agonist that gives a response halfway between top and bottom of the curve and were calculated as part of the curve fitting. Data were analysed for statistical significance using one way or two-way ANOVA with post hoc test as appropriate. Significance was taken as $P<0.05$. 
CHAPTER 3

EFFECT OF TETRACYCLINE DERIVATIVES ON PURINERGIC RECEPTORS

3.1 Introduction

Minocycline: an anti-inflammatory and neuroprotective tetracycline antibiotic

Minocycline (MINO) is a second generation, semi-synthetic, broad-spectrum bacteriostatic antibiotic derived from four carbon rings containing polyketide, tetracycline, a naturally occurring antibiotic (Figure 3.1) (244, 245). The antibiotic properties of MINO can be attributed to its ability to inhibit protein synthesis in bacteria by binding to the 30S ribosomal subunit, thereby preventing the tRNA from binding to the ribosomal complex (246). MINO exhibits a good pharmacokinetic profile with rapid and complete absorption, almost 100% bioavailability and a long half-life (247-249). It is used clinically for the treatment of acne vulgaris, some sexually transmitted diseases and rheumatoid arthritis (250-253). It is well tolerated by individuals of all ages and has a good safety profile, which makes it a recommended drug by US FDA (Food and Drug Association, United States) (254).

![Chemical structure of MINO](image)

Figure 3.1 Chemical structure of MINO (C\textsubscript{23}H\textsubscript{27}N\textsubscript{3}O\textsubscript{7})

More recently MINO has attracted interest because of its anti-inflammatory and anti-apoptotic actions independent of its antibiotic properties. It is highly lipophilic allowing it to cross the
blood-brain barrier and inhibit microglial activation (255-258), amyotrophic lateral sclerosis (259-262), depression (263, 264), Parkinson’s disease (265-268), schizophrenia (269), stroke, autism and fragile X-syndrome (254, 270-272). Furthermore, MINO is under clinical assessment as an adjuvant treatment for bipolar depression (271, 273), multiple sclerosis (274), bipolar disorders (275) and schizophrenia (276). It is in early clinical trials for schizophrenia, ischaemic stroke and pain (277-279). Meta-analysis of randomised clinical trials before September 2017 has indicated that MINO has a significant anti-depressant effect and good tolerance in comparison to a placebo in humans (271). A randomised, double-blind placebo-controlled trial of MINO on at-risk mental state (ARMS) people has indicated the beneficial effects of this anti-inflammatory drug in delaying the manifestation of psychosis (280).

The mammalian CNS consists of two main cell types: neurons and glia. Glial cells are subdivided into microglia, Bergmann cells (cerebellum) and Müller cells (only in the retina) (281). Microglia are the immunomodulatory cells of central nervous system (282). In case of injury or insult to the CNS, microglia become activated and change into motile, phagocytic and cytotoxic cells, which infiltrate the site of injury (283). The levels of ATP around glial cells can increase due to either neuronal release from synapses and along axons, from astrocytes via P2 purinergic receptors or through cellular damage (284). The elevated levels of ATP activate the purinergic receptors in microglia leading to increases in intracellular Ca$^{2+}$ (285). The Ca$^{2+}$ influx further affects a number of downstream pathways leading to inflammation or apoptosis (286). In vitro studies have shown that MINO can block LPS-induced inflammatory cytokine release (287-289). In one study, IFN-α was used to induce depression-like behaviour in mice and it was observed that MINO was able to downregulate these pro-inflammatory signals, thereby alleviating symptoms of depression (290). Microglial activation and glutamate receptor-1 phosphorylation has been observed in the brains of chronically stressed mice and the cognitive disturbances were seen to be ameliorated by MINO treatment (291). Microglial
Activation has also been observed in schizophrenia and MINO administration attenuated microglial proliferation and behavioural symptoms in a mouse model of schizophrenia (292).

The basic hypothesis tested by many of the studies mentioned above is that microglial activation and proliferation occur in the brain during stress, injury or ischemia. The activated microglia produce inflammatory cytokines and these inflammatory signals can ultimately cause irreparable neuronal cell injury and MINO can prevent this by inhibiting the microglial activation (Figure 3.2) (254).

![Figure 3.2 A schematic diagram displaying P2X7 and associated signalling pathways.](image)

MINO may affect the P2X7 channel activity directly or the associated membrane pores in order to inhibit the downstream signalling pathways, thereby preventing pro-inflammatory responses and apoptosis.
Possible molecular targets of MINO

Many of the reported cellular actions of MINO include inhibition of protein kinase C (289, 293), p38 and other MAP kinases (294-296), cytochrome c (297) along with cytokine release, nitric oxide and ROS production, as reviewed in (279, 298). There is some evidence of MINO directly targeting membrane metalloproteinases (MMP) thereby preventing the T- lymphocytes from migrating through the fibronectin matrix, making MINO efficacious in treating diseases such as multiple sclerosis, cerebral haemorrhage, encephalitis and Fragile X-syndrome (299). Nanomolar concentrations of MINO have been seen to successfully inhibit poly(ADP-ribose) polymerase-1 (PARP-1), the enzyme involved in DNA damage-induced cell death and inflammation, providing some explanation to the anti-inflammatory and neuroprotective activities of MINO (300, 301). However, it is currently unclear whether direct inhibition of PARP-1 underlies the clinical effects of MINO. Besides PARP-1, MINO has not yet been shown to act directly on most of the reported targets and therefore inhibition of such signalling pathways may be indirect. Indeed, MINO may act on multiple targets to evoke its neuroprotective action (254).

As mentioned earlier, microglia are known to endogenously express a heterogeneous collection of purinergic receptors including ionotropic P2X7, P2X4 as well as metabotropic P2Y receptors (117-119, 302). The expression of purinergic receptor P2X7 is known to increase in activated microglia (303). These ATP-activated ion channels are coupled to many of the intracellular signalling events that MINO is reported to block including microglial activation and proliferation (116), ROS production (158), apoptosis (304) and cytokine secretion (304). Furthermore, P2X7 and P2X4 receptors have been implicated in the pathology of many of the diseases for which MINO has reported efficacy (305, 306). In a study conducted by Itoh et al., activation of P2X7 receptors in vivo was involved in the development of central sensitization in an acute inflammatory pain model. In this study, the activation of P2X7 by specific agonists
led to an increase in central sensitization and blocking by P2X7 inhibitors and MINO reduced this inflammatory pain (307). From this study, it was concluded that the P2X7 receptors are mainly expressed on the microglia in the medullary dorsal horn and that they play a central role in nociceptive responses. In another study investigating the rat model of chronic prostatitis, rats induced with the disease had higher levels of inflammatory cytokines and blocking of microglia by MINO or a P2X7 antagonist downregulated this cytokine production. The results from this study suggested that microglia and P2X7 activation are involved in pain associated with chronic prostatitis (308).

### 3.2 Rationale for the research

A large array of research indicates that MINO acts on microglia and downregulates inflammatory cytokine production. P2X7 receptors are located on glia and are involved in chronic inflammatory signalling pathways. Therefore, this project investigated whether MINO’s inhibitory action on microglial activation works through direct blocking of the purinergic P2X7 ion channels or the associated signalling pathways.

In order to attain a better insight into the selectivity and structure-function relationship of the drugs towards these ion channels, the effects of tetracycline and two of its other derivatives (doxycycline and tigecycline) on P2X7 and its close relative, P2X4 were also been tested.

The data from this study may help elucidate the underlying mechanism of action of MINO in a variety of neurological diseases and conditions.

### 3.3 Research Question

The present study tested the hypothesis that MINO may be eliciting its inhibitory effect on microglial activation and proliferation via purinergic ion channels, P2X7 and P2X4.
3.4 Results

3.4.1 P2X7 responses in BV-2 microglia

Minocycline (MINO) is known to inhibit microglial activation in vivo and in vitro (309, 310). In the present study, the BV-2 mouse microglial cell line was used to investigate ATP-induced responses and the effect of MINO. First, an ATP-mediated dye uptake assay was utilised to measure P2X responses in BV-2 cells and the increase in YO-PRO™-1 Iodide (YOPRO-1) fluorescence over time was inhibited with AZ10606120 (Figure 3.3 A), a selective P2X7 inhibitor (211). It was found that MINO effectively reduced ATP-induced responses in BV-2 cells at concentrations >50 µM (Figure 3.3 B and Figure 3.4). Intracellular Ca\(^{2+}\) responses were measured to ATP in Fura-2 AM loaded BV-2 cells using a plate-reader based assay. Intracellular Ca\(^{2+}\) increased with time after the application of ATP (Figure 3.5). This ATP-induced Ca\(^{2+}\) response was a P2X7-mediated response as the sustained phase of Fura-2 fluorescence was abolished in the presence of the P2X7 antagonist AZ10606120 (10 µM). It was observed that MINO significantly reduced the sustained Ca\(^{2+}\) responses in BV-2 microglia.
Figure 3.3 Effect of MINO on P2X7 mediated YOPRO-1 uptake in BV-2 microglial cells

(A) YOPRO-1 uptake was measured in BV-2 cells as an increase in fluorescence after agonist application at 40 seconds on a Flexstation 3. Cells were pre-incubated with MINO for 30 minutes at 37 °C before ATP (1 mM) injection. (B) The dye uptake was quantified as area under the dye uptake curve and plotted as bar graph. Control YOPRO-1 uptake to ATP (100 µM and 1 mM) in the absence of drugs is shown in black bar, 10 µM AZ10606120 is shown in white open bar, in presence of 50 µM is shown in light grey bar and in the presence of 100 µM MINO is shown in dark grey bar. Bars represent mean data from 18-20 wells from 2–3 separate experiments. Data was calculated as area under the normalised curve (50-300 seconds). Error bars represent S.E.M, * P<0.05 calculated by one-way ANOVA (Dunnett’s post-test).
Figure 3.4 Effect of MINO on P2X7 activated by various concentrations of ATP in BV-2 microglial cells

Dye uptake was induced by stimulating cells with different concentrations of ATP (1mM, 500 μM, 250 μM, 100 μM, 50 μM, 25 μM, 10 μM and 1 μM) in low divalent cation solution on a Flexstation 3. The fluorescence was measured for 300 seconds and non-linear fit of transformed responses were fitted in GraphPad Prism. MINO at 100 μM concentration increases the EC$_{50}$ of ATP for P2X7 activation from 184.1 μM to 243.6 μM (95% confidence interval- 300.9 to 701.2). The data was collated from 3 different experiments and the dose response curves were generated using non-linear fit of transformed data on GraphPad prism software. Error bars represent S.E.M.
Figure 3.5 Effect of MINO on P2X7 mediated Ca\(^{2+}\) influx in BV-2 microglial cells

(A) Intracellular Ca\(^{2+}\) responses were measured in Fura-2 AM loaded BV-2 cells using a Flexstation 3 plate reader. Fura-2 ratio (340nm/380nm) was normalised to zero baseline. ATP (1mM) was injected after 30 seconds as indicated by the arrow. (B) represents the effect of different drugs on the sustained phase of the response to 1mM ATP. Bar charts represent mean data from 24-32 wells from 4 separate experiments. Ca\(^{2+}\) in the presence of P2X7 agonist ATP is shown here in black bar, in the presence of 100 µM, 50 µM and 10 µM MINO are represented by grey bars. The ATP-mediated Ca\(^{2+}\) response was inhibited by AZ10606120. Data was calculated as mean of sustained responses (100-300 seconds); baseline corrected. Error bars represent S.E.M, * denotes P<0.05 calculated by one-way ANOVA (Dunnett’s post-test). The concentration of ATP added to all treatments is 1 mM.
Figure 3.6 Dose dependent inhibitory effect of MINO on P2X7 mediated Ca\textsuperscript{2+} influx in BV-2 microglial cells

The inhibitory effect of MINO on the sustained phase of the Ca\textsuperscript{2+} response to 1 mM ATP in the presence of various concentrations of MINO was measured using Ca\textsuperscript{2+} sensitive ratiometric dye Fura-2 AM. BV-2 microglia pre-treated with various concentrations of MINO (0.1-100 µM) were activated with 1 mM ATP. Fura-2 fluorescence was measured on Flexstation 3. The IC\textsubscript{50} was calculated from the non-linear fit of transformed data. IC\textsubscript{50} of MINO was found to be 6.5 µM (95% confidence interval - 0.1 to 1.8). Data collated from 3 independent experiments. Error bars represent S.E.M.
3.4.2 P2X7-mediated pro-inflammatory responses

P2X7 mediates a number of pro-inflammatory events such as release of IL-1 β from ATP activated immune cells (311). In Figure 3.7, the effect of MINO was evaluated on P2X7-mediated IL-1β release in LPS-primed BV-2 microglia and it was found that 100 µM MINO pre-treatment was able to reduce ATP-induced IL-1β release in LPS-primed microglial cells. An increase in IL-1β levels was observed in ATP treated cells in comparison to control cells. No significant difference in IL-1β levels was observed between LPS primed and non-primed cells. Multiple comparisons using one-way ANOVA showed ~ 30% decrease in IL-1β release between the MINO treated LPS primed ATP activated cells and the non-MINO treated cells. These results indicate that MINO has an inhibitory effect on pro-inflammatory responses mediated by P2X7. MINO is known to inhibit microglial activation and proliferation (312). In Figure 3.8, the effect of varying concentrations of MINO on proliferation rates in BV-2 cells was measured using the CellTiter 96® AQueous One Solution assay. BV-2 microglia in the presence of MINO (100 µM and 50 µM; 24 hours) showed a significant decrease in cell number in comparison to untreated controls. This effect could be P2X7-mediated as A438079 (a P2X7 specific antagonist) also reduced BV-2 cell number in comparison to untreated controls (Figure 3.8). The reduction in cell number was also observed in the presence of the P2X4 antagonist 5-BDBD (Figure 3.8) but the cells showed some morbid changes in morphology suggesting the involvement of toxicity.
Figure 3.7 Effect of MINO on IL-1β release in BV-2 microglial cells as measured by ELISA

IL-1β levels in each treatment group were measured using a Thermo Fisher Mouse-IL-1β kit using manufacturer’s instructions. The IL-1β levels in untreated controls are shown as pale pink while the orange bar represents IL-1β secretions LPS treated cell. IL-1β production in LPS primed cells activated by 500 µM ATP is shown in black while IL-1β production by cells activated with LPS + 500 µM ATP is shown in orange with black dots. The IL-1β production is LPS + ATP treated cells is reduced by treatment with 100 µM MINO (dark grey bar). The P2X7 antagonist AZ438079 (light grey bar), however, does not significantly reduce IL-1β release under these conditions. The bar graph was generated from collated data from 3 separate experiments (n=3). The IL-1β release was quantified using linear regression equation from a standard curve. Error bars represent S.E.M, * P<0.05 calculated by one-way ANOVA (Dunnett’s multiple comparison test).
Figure 3.8 Effect of MINO on cell proliferation in BV-2 microglial cells as measured by Cell Titer 96® AQueous One Solution Assay

CellTiter 96® AQueous One Solution Reagent was used to measure cell viability after 24 h treatment period. The absorbance was read at 490 nm on a Clariostar microplate reader. Bar charts represent mean data from 9-12 wells from 3 separate experiments. Data was calculated by normalising absorbance of media to 100% and the other conditions are plotted as percentage of media control. The black bar represents the absorbance of untreated control cells (normalised to 100%). 0.01 % DMSO has no significant effect on proliferation rates (black chequered bar). The effect could be P2X7 mediated as a reduction in cell number is seen in the presence of AZ438790 (P2X7 antagonist), shown in white open bar. Reduction in cell number is also seen with P2X4 antagonist 5-BDBD (light grey bar) but the cells exhibited some abnormal morphological changes suggesting mechanisms other that inhibition in cell proliferation. The grey bars represent effect of MINO (different concentrations) and it was seen that MINO decreases cell proliferation rates in a concentration dependent manner. Error bars represent S.E.M. * denotes $P<0.05$ calculated by one-way ANOVA (Dunnet’s multiple comparison test).
3.4.3 Investigating the selectivity of MINO

To investigate these channels individually, recombinant hP2X7 or hP2X4 were expressed in HEK-293 cells and functional responses were measured using the YOPRO-1 dye uptake assay (147, 313). The use of such dye uptake assays to measure P2X4 responses is less well defined in the literature, however, ATP-induced activation of P2X4 does lead to a secondary permeability state (314) and Bernier et al have demonstrated YOPRO-1 iodide uptake in P2X4-expressing HEK-293 cells (315). The effect of MINO was first investigated on rat P2X7 (Figure 3.9) and rat P2X4 (Figure 3.10) expressed in HEK-293 cells as preliminary data obtained in our laboratory on rat primary microglial cells demonstrated an effect of MINO on ATP-induced dye uptake both in the absence or presence of AZ10606120 (a P2X7 antagonist) or MINO (Jack Watson, Martin Stebbing, personal communication). Different concentrations of ATP were used to stimulate the two P2X channels; 1 mM ATP for rat P2X7 and 10 µM ATP for rat P2X4. AZ10606120 (10 µM) completely abolished the response to ATP only in cells expressing rat P2X7 (Figure 3.9). AZ10606120 had no effect on rat P2X4 responses, demonstrating that it is not a P2X7 response (Figure 3.10). MINO (100 µM) significantly reduced responses at both rat P2X7 and rat P2X4. One-way ANOVA analysis for rat P2X7 responses revealed a 69.5 % decrease between dye uptake in presence of ATP (1 mM) and ATP + 100 µM MINO. This was a P2X7 mediated response as P2X7 specific antagonist AZ10606120 decreased the ATP responses by 96.8 %. Similarly, 100 µM MINO inhibited ATP (10 µM) responses in HEK- rat P2X4 by 68.8 %.

The effect of MINO was further investigated on human P2X7 (hP2X7) and human P2X4 (hP2X4) as it is well known that many compounds can have substantial species-selective effects on P2X7 (211) and very little is known about human P2X4 pharmacology. AZ10606120 effectively inhibits rat, mouse and human P2X7 (211, 316) and clearly abolished hP2X7 responses (Figure 3.10). ATP-induced dye uptake via human P2X7 was substantially reduced
(~ 70 % decrease) by 100 µM MINO (Figure 3.11). To test the its selectivity for P2X7, MINO was tested for its effect on closely related purinergic receptor, P2X4. HEK-293 cells expressing hP2X4 displayed ATP-induced dye uptake that was not blocked by AZ10606120 (10 µM). MINO (100 µM) showed a significant inhibition of hP2X4 responses (Figure 3.12). Following the establishment that MINO inhibited both P2X7 and P2X4 mediated YOPRO-1 uptake, a comparison between the magnitudes of inhibitory effects of MINO for each of these receptors was done by comparing the area under the dye uptake curves from the above experiments (Figure 3.11). Multiple comparisons using one-way ANOVA indicated a decrease of 76.11 % between dye uptake in ATP treated and ATP + 100 µM MINO treated human P2X7 expressing HEK-293 cells. Even though, MINO was found to inhibit dye uptake in both HEK-P2X7 and HEK-P2X4, the amount of inhibition was more for P2X7 receptor as compared to P2X4 responses. This was found out by comparing the dye uptake data from the two types of receptors tested (Figure 3.12). For comparing the level of inhibition caused by MINO for P2X7 and P2X4 mediated dye uptake, the area under the dye uptake curve for controls was normalised to 100 % and dye uptake by MINO treated cells was plotted as percentage of control. It was found that 100 µM MINO was able to inhibit ~ 70 % of the dye uptake caused by ATP in HEK-P2X7 cells while the % inhibition caused by 100 µM MINO in HEK-P2X4 cells was ~ 45 %. Thus, MINO had a larger effect on P2X7 in comparison to P2X4 (Figure 3.13).
Figure 3.9 Effect of MINO on YOPRO-1 uptake in HEK-293 cells expressing rat P2X7

YOPRO-1 uptake was measured in HEK-rP2X7 expressing cells as an increase in fluorescence after agonist application at 40 seconds on a Flexstation 3. Cells were pre-incubated with MINO for 30 minutes at 37 °C before 1 mM ATP injection. (A) The raw traces of dye uptake from Flexstation 3 are shown here. The fluorescence increased immediately following injection of 1 mM ATP on to HEK-293 cells expressing rat P2X7 at 40 seconds (black circles) and is inhibited by MINO (charcoal grey circles) and P2X7 antagonist AZ10606120 (light grey circles). (B) The dye uptake was quantified as the area under the dye uptake curve and plotted as a bar graph. Control YOPRO-1 uptake in response to ATP in the absence of drugs is shown in black, and P2X7 antagonist 10 μM AZ10606120 is shown in grey bar, in the presence of 10 μM MINO is shown in light grey bar and the 100 μM MINO is shown in white. Bar charts represent mean data from 18-20 wells from 2–3 separate experiments. Data was calculated as area under the normalised curve (50-300 seconds). Error bars represent S.E.M, *represents $P<0.05$ calculated by one-way ANOVA (Dunnett’s post-test).
Figure 3.10 Effect of MINO on YOPRO-1 uptake in HEK-293 cells expressing rat P2X4

YOPRO-1 uptake was measured in HEK-rP2X4 expressing cells as an increase in the fluorescence after agonist application at 40 seconds on a Flexstation 3. Cells were pre-incubated with MINO for 30 minutes at 37 °C before 10 µM ATP for P2X4 injection. (A) represents the raw traces from a Flexstation 3. The fluorescence increases immediately following the injection of 10 µM ATP on to HEK-P2X4 cells at 40 seconds (black circles) and was inhibited by MINO (grey circles). (B) Quantification of dye uptake was done as the area under the dye uptake curve and plotted as bar graph. Bar charts represent the mean of data from 18-20 wells from 2–3 separate experiments. Dye uptake in the presence of 10 µM ATP is shown as a black bar, in the presence of ATP + 10 µM MINO is shown in light grey and in the dark grey. Data was calculated as the area under the normalised curve (50-300 seconds). Error bars represent S.E.M. * P<0.05 calculated by one-way ANOVA (Dunnett’s post-test).
Figure 3.11 Effect of MINO on ATP-induced dye uptake in HEK-293 cells stably expressing human P2X7

YOPRO-1 uptake was measured in HEK-hP2X7 pre-incubated with MINO for 30 minutes at 37 °C before 1 mM ATP injection. The raw traces of dye uptake from a Flexstation 3 are shown here. The fluorescence increased immediately following the injection of 1mM ATP on to HEK-P2X7 cells at 40 seconds (black circles) and was inhibited by MINO (grey circles) and P2X7 antagonist AZ10606120 (white circles). (B) The dye uptake was quantified as the area under the dye uptake curve and plotted as a bar graph. Bar charts represent the mean of data from 18-20 wells from 3 separate experiments. Black bar represents dye uptake in the presence of 1 mM ATP, white (+ grey error bar) represents AZ10606120, ATP + 10 µM MINO is shown in light grey and ATP + 100 µM MINO is shown in dark grey bar. Data was calculated as the area under the normalised curve (50-300 seconds). Error bars represent S.E.M, * P<0.05 calculated by one-way ANOVA (Dunnett’s post-test).
Figure 3.12 Effect of MINO on ATP-induced dye uptake in HEK-293 cells stably expressing human P2X4

YOPRO-1 uptake was measured in HEK-hP2X4 expressing cells as an increase in fluorescence after agonist application at 40 seconds on a Flexstation 3. Cells were pre-incubated with MINO for 30 minutes at 37 °C before addition of 10 µM ATP. (A) The raw traces of dye uptake from a Flexstation 3 are shown here. The fluorescence increased immediately following injection of 1 mM ATP on to HEK-P2X4 cells at 40 seconds (black circles) and was inhibited by MINO (charcoal grey circles). (B) The dye uptake was quantified as the area under the dye uptake curve and plotted as a bar graph. Bar charts represent the mean of data from 18–20 wells from 2–3 separate experiments. Data was calculated as the area under the normalised curve (50–300 seconds). Error bars represent S.E.M, * P<0.05 calculated by one-way ANOVA (Dunnett’s post-test).
MINO attenuates the ATP induced dye uptake or macropore formation mediated by both human P2X7 and P2X4, but the magnitude of this reduction is greater in HEK-293 cells expressing P2X7 in comparison to cells HEK-293 expressing P2X4 receptors. This graph represents the dye uptake by ATP activated HEK-P2X7 and HEK-P2X4. The black bars represent the dye uptake by control cells in the presence of ATP only (1 mM for P2X7 and 10 µM for P2X4). The open black bars represent dye uptake in cells pre-treated with 100 µM MINO for 30 minutes and then activated by ATP. For comparison, the area under the dye uptake curve for controls was normalised to 100 % and dye uptake by MINO treated cells was plotted as percentage of control. Error bars represent S.E.M, * \( P<0.05 \) calculated by one-way ANOVA (Dunnett’s post-test).

Figure 3.13 Comparison of the effect of MINO on ATP-induced dye uptake in HEK-293 cells stably expressing human P2X7 or human P2X4.
3.4.4 Investigating internalisation of P2X7 receptors

It is known that P2X4 channel responses can desensitize rapidly via agonist-induced internalisation of receptors (317), therefore, the possibility of MINO stimulating internalisation of P2X7 receptors was investigated. When the surface expression of P2X7 was quantified using an extracellular domain-binding antibody (clone L4) and live cell flow cytometry, there was no effect of 100 µM MINO on P2X7 expression (Figure 3.14 B). This suggested that stimulation of internalisation was not the mechanism by which MINO was affecting dye uptake responses mediated by P2X7 receptors.

3.4.5 Investigating the effect of MINO on YOPRO-1 fluorescence

To ensure that the inhibition of fluorescence exhibited by MINO was an actual effect on the channel and not quenching of fluorescence by the drugs, HEK-hP2X7 cells were incubated with ATP (1 mM or 100 µM) for 5 - 10 minutes at 37 ºC to allow YOPRO-1 to be taken up by the cells. Fluorescence was recorded using the Flexstation and MINO was injected to a final concentration of 100 µM. If MINO was having a quenching action on fluorescence, it was expected that the fluorescent signal would decrease. The data show that there was no decrease in fluorescence signal following MINO injection (Figure 3.14 A). To further ensure that MINO was not directly acting on the YOPRO-1 fluorescence, dye uptake was measured for HEK-293 cells pre-incubated with 100 µM and then lysed with a low concentration of detergent, 0.01 % Triton X-100. The rationale for this assay was that if P2X receptors were not required for MINO action, then MINO would be able to act on YOPRO-1 bound to DNA of lysed cells, thereby decreasing the YOPRO-1 fluorescence. The results from these experiments (Figure 3.15) show that MINO is unable to cause any decrease in maximum YOPRO-1 fluorescence of lysed cells, thus indicating that the presence of a specific receptor is required for MINO action.
Figure 3.14 MINO does not quench fluorescence of YOPRO-1 dye and does not alter receptor expression on the cell surface.

(A) The effect of MINO on fluorescence of the YOPRO-1 dye was performed by incubating HEK-hP2X7 cells with ATP (1 mM or 100 μM) for 5-10 minutes at 37 °C and then injecting MINO to a final concentration of 100 μM. The treatment of cells with MINO does not show any reduction in fluorescent signal. The bar charts represent mean data from 6 wells from 2 separate experiments. The black bars represent fluorescence in presence of ATP alone and grey bars represent fluorescence in presence of ATP + MINO. Data was calculated at 41-60 second time point as baseline corrected endpoint data. (B) The effect of MINO on expression of P2X7 was measured by flow cytometer with HEK-hP2X7 stable cells. Cells were treated with ATP + 100 μM MINO (red trace) or ATP alone (black trace) for 30 minutes and then detached with 0.05 % Trypsin EDTA. The cells were washed with 0.5 % BSA in 0.1 mM PBS and then incubated on ice with an anti-P2X7-FITC (1:100) antibody or isotype control for 1 hour. Staining was measured using a BD FACS Canto flow cytometer.
Figure 3.15 MINO does not reduce the maximum YOPRO-1 uptake in lysed cells

Triton X-100 (0.01 %) was used to lyse the cell membrane in order to obtain maximum dye uptake. HEK-P2X7 cells pre-incubated with 100 µM MINO did not show inhibition in dye uptake providing evidence that MINO acts via P2X mediated responses and not on fluorescence of the dye itself. Bars represent mean data of 12 wells for 2 separate experiments. Black bar represents YOPRO-1 fluorescence in the presence of 0.01 % Triton X-100 and grey bar represents fluorescence in presence of 0.01 % Triton X-100 + 100 µM MINO. Data was quantified as area under dye uptake curve (baseline corrected). Error bars represent S.E.M, ns denotes not significant, $P<0.05$ calculated by one-way ANOVA (Dunnett’s post-test).
3.4.6 Investigating the pharmacological properties of MINO towards P2X7 and P2X4.

The pharmacological measurements were further extended to perform concentration-responses for ATP in the absence or presence of increasing concentrations of MINO (10, 50, 200 µM) for both human P2X7 (Figure 3.16) and human P2X4 (Figure 3.17). MINO showed a non-competitive inhibition of ATP responses at both P2X receptors and did not fully inhibit responses at either channel at concentrations up to 200 µM. The IC$_{50}$ value for the inhibitory action of MINO for hP2X7 was found to be 23.2 µM (95 % confidence interval - 14.5 to 36.8). The IC$_{50}$ value for MINO in hP2X4 was found to be 53.8 µM (95 % confidence interval - 32.4 to 89.5).
A concentration-response curve for ATP-induced YOPRO-1 uptake in a HEK-hP2X7 stable cell line in the absence and presence of increasing concentration of MINO (10 – 200 µM) was generated. MINO decreased the amplitude of ATP responses with no obvious competitive effects. Dose responses were generated as non-linear fit of transformed data using GraphPad Prism software. EC$_{50}$ for ATP at hP2X7 was 116.9 µM (95 % confidence interval 164.1 – 533.8 µM). (B) An IC$_{50}$ plot for MINO acting on human P2X7 using a maximum concentration of ATP (500 µM). Experiments were performed on 4 separate occasions and data collated. Dose responses were generated as non-linear fit of transformed data using GraphPad Prism software. IC$_{50}$ for MINO was 32.6 µM (95 % confidence interval 7.9 – 135 µM).
Figure 3.17 MINO acts as a non-competitive antagonist at human P2X4 receptors

(A) Concentration-response curve for ATP-induced YOPRO-1 uptake in a HEK-hP2X4 stable cell line in the absence and presence of increasing concentration of MINO (0.01 – 10 µM). MINO decreases the amplitude of ATP responses with no obvious competitive effects. The EC$_{50}$ for ATP at hP2X4 was 0.2 µM (95 % confidence interval 0.2 – 0.3 µM). (B) An IC$_{50}$ plot for MINO acting on human P2X4 using a maximum concentration of ATP (10 µM). Experiments were performed on 4 separate occasions and data collated. Dose responses were generated as non-linear fit of transformed data using GraphPad Prism software. IC$_{50}$ for MINO was 53.8 µM (95 % confidence interval 32.4 – 89.4 µM).
3.7 Investigating the effect of tetracycline and its structural analogues on P2X7

Investigations were made into the action of three other tetracycline antibiotics on P2X receptors. The parent compound tetracycline (TET) and the clinically relevant antibiotics doxycycline (DOX) and tigecycline (TIGE) were chosen. DOX is a second-generation tetracycline class antibiotic also known to have inhibitory effects on microglia and anti-inflammatory effects in diseases such as rheumatoid arthritis, COPD and Lyme disease (276, 318, 319). DOX displayed a similar inhibitory effect on hP2X7 (Figure 3.18) whereas TET and TIGE had no inhibitory effect (Figure 3.18). There is evidence that DOX is as effective as MINO in its anti-inflammatory properties. DOX is said to be clinically more suitable as it does not exhibit any common side effects associated with MINO usage (320-322). Keeping this in mind, the pharmacology of DOX was further investigated on P2X7 responses. A concentration-dependent response of DOX on hP2X7 responses was performed (Figure 3.19) and DOX was found to non-competitively inhibit hP2X7 responses with an IC$_{50}$ of 36.9 µM (95 % confidence interval 10.7 – 127 µM).
Figure 3.18 Effect of tetracycline class antibiotics on ATP-induced dye uptake in HEK-P2X7.

YOPRO-1 uptake was induced in HEK-hP2X7 expressing cells by the addition of 1 mM ATP in low divalent physiological solution. Drugs (MINO, DOX, TIGE and TET) were prepared in low divalent buffer containing 2 μM YOPRO-1. Cells were pre-incubated with drugs for 30 minutes at 37 °C before ATP injection on Flexstation 3. DMSO (0.01 %) was added as vehicle control for TIGE and TET. The bar charts represent mean data from 25-30 wells from 4-5 separate experiments. The effects of MINO on P2X7 responses is shown in panel A, TIGE is shown in panel B, DOX is shown in panel C and TET responses are shown in D. Data was calculated as the area under the dye uptake curve at 50-300 second time point (baseline corrected). Error bars represent S.E.M, ** denotes $P<0.05$ calculated by one-way ANOVA (Dunnett’s post-test).
Figure 3.19 TET analogue DOX also attenuates P2X7 mediated responses

(A) Concentration-dependent responses were measured for ATP-induced YOPRO-1 uptake in a HEK-hP2X7 stable cell line ± DOX (10 – 200 µM). DOX reduced the amplitude of ATP responses with no obvious competitive effects. EC$_{50}$ for ATP at hP2X7 was 110.8 µM. An IC$_{50}$ plot for MINO acting on human P2X7 using a maximum concentration of ATP (500 µM). The dose response curves were generated as non-linear fit of transformed data using GraphPad prism software. Experiments were performed on 4 separate occasions and data was collated. The IC$_{50}$ for MINO was 36.9 µM (95% confidence interval 10.7 – 127 µM).
3.8 Validating the effect of MINO and DOX on P2X7 responses using primary macrophages

In order to further validate the above results, the effect of MINO and DOX on P2X7 responses was investigated in primary macrophages isolated from the peritoneum of Wistar rats. Macrophages, just like microglia, endogenously express the purinergic receptors P2Y1, P2Y2, P2Y4, P2Y11-14, P2X4, and P2X7 (323). Various concentrations of DOX and MINO were tested on primary macrophages activated by 500 µM ATP. It was observed that both MINO and DOX dose dependently inhibited rat P2X7 mediated Ca$^{2+}$ influx in Fura-2 AM loaded cells. The IC$_{50}$ for DOX in rat macrophages is 16.0 µM (95 % confidence interval 1.8-14 µM). The IC$_{50}$ for MINO in rat macrophages is 19.1 µM (95 % confidence interval 4.7-77 µM) (Figure 3.20).
Figure 3.20 MINO and DOX have an inhibitory effect on P2X7 mediated Ca\(^{2+}\) influx in primary peritoneal macrophages

Concentration response effects were measured for MINO and DOX acting on human P2X7 in primary peritoneal macrophages activated by 500 µM ATP. Experiments were performed on 2 separate occasions using macrophages isolated from 3 Wistar rats per experiment and data was collated. The Ca\(^{2+}\) influx was measured by loading the cells with ratiometric Ca\(^{2+}\) indicator Fura-2 AM. The fluorescence was measured on a Flexstation 3 immediately following the injection of ATP in cells pre-incubated with MINO or DOX. (A) The graph represents the non-linear transform of normalised sustained Ca\(^{2+}\) response (100-300 seconds) in rat macrophages pre-incubated for 15-30 minutes with varying concentrations of MINO (1µM – 100 µM). The IC\(_{50}\) for MINO was 19.09 µM (95 % confidence interval 4.7 – 77 µM). (B) The graph represents the non-linear transform of normalised sustained Ca\(^{2+}\) response (100-300 seconds) in rat macrophages pre-incubated for 15-30 minutes with varying concentrations of DOX (1 µM-100 µM). The IC\(_{50}\) for DOX was found to be 16.04 µM (95 % confidence interval 1.8–14 µM).
3.4.9 Investigating the effect of MINO and DOX on P2Y1 and P2Y2 receptors

To establish the selectivity of MINO towards purinergic receptors expressed in microglia, the effect of MINO (100 µM) was tested on non-transfected HEK-293 cells endogenously expressing P2Y1 and P2Y2 receptors. The receptors were activated by the P2Y1 agonist ADP, and the P2Y2 agonists, ATP and UTP (10 µM for each). The effect of pre-incubated MINO was measured on P2Y1 or P2Y2-mediated Ca\(^{2+}\) responses in HEK-293 cells using Fura-2 AM. The peak responses were measured, and it was observed that MINO has a significant inhibitory effect on both P2Y1 as well P2Y2 mediated Ca\(^{2+}\) release from intracellular stores (Figure 3.21). A mean difference in Fura-2 fluorescence of 97.2 (95 % confidence interval 86 to 108) was calculated between ATP and ATP + 100 µM MINO treated cells. The mean difference of Fura-2 fluorescence for ADP activated HEK-293 cells, was found to be 96.9 (95 % confidence interval 85.9 to 108.1). Finally, the mean difference between UTP and UTP + MINO Ca\(^{2+}\) response was 61.5 (95 % confidence interval 48.6 to 74.4). These results indicate that MINO inhibits the P2Y mediated release of Ca\(^{2+}\) from intracellular stores.
Figure 3.21: MINO inhibits P2Y1 and P2Y2 mediated intracellular Ca^{2+} influx in HEK-293 cells

The effect of MINO on P2Y1 and P2Y2 mediated Ca^{2+} responses in HEK-293 cells endogenously expressing these receptors were investigated. The HEK-293 cells were loaded with 2 µM Fura-2 AM Ca^{2+} indicator and stimulated with either 10 µM ATP (P2Y1, P2Y2 agonist) or 10 µM ADP (P2Y1 agonist) or 10 µM UTP (P2Y2 agonist). Responses were measured as peak Ca^{2+} influx from 0-100 sec. Bars represents mean of normalised data from 18-20 wells from 3 separate experiments. Data was calculated as maximum peak value (baseline corrected). Error bars represent S.E.M, * P<0.05 calculated by two-way ANOVA (Sidak’s multiple comparison test).
3.4.10 Investigating the effect of MINO and DOX on TRPV-1 ion channels

The above results indicate that MINO (as well as DOX) may inhibit P2X mediated membrane pore formation. Even though membrane pore formation is a characteristic feature of P2X7 receptor activation, there are other membrane receptors, which display similar properties. One such ion channel is TRPV-1 (324). To evaluate whether MINO affects P2X membrane specifically, drugs were tested on T-REx- HEK-293 cells stably expressing rat TRPV-1 using the YOPRO-1 dye uptake assay (Figure 3.22). T-REx- HEK rat TRPV-1 cells were plated overnight. The cells were stimulated with 1µg/ml tetracycline for 4 hours in CO₂ incubator. YOPRO-1 was induced by the addition of 100 nM capsaicin in low divalent physiological solution. MINO (100 µM) was prepared in low divalent buffer containing 2 µM YOPRO-1. Cells were pre-incubated with MINO for 30 minutes at 37 °C before application of agonist ATP using Flexstation 3. DMSO (0.01 %) was added as vehicle control. Capsaicin activated TRPV-1 membrane formation (indicated by YOPRO-1 uptake) which was effectively attenuated by the TRPV-1 antagonist CPZ. MINO (100 µM) also inhibited rat TRPV-1 responses to capsaicin (Figure 3.22). A mean decrease of 51.9 % dye uptake was observed in rat TRPV-1 pre-incubated with MINO before activation by capsaicin.
Figure 3.2: MINO inhibits dye uptake in T-Rex-HEK rat TRPV-1 cells

The effect of MINO on capsaicin-induced dye uptake in T-Rex-HEK-293 cells stably expressing rat TRPV-1 was investigated. The bar charts represent mean data from 12-15 wells from 3 separate experiments. Data was calculated as the area under the dye uptake curve from 50-300 second (baseline corrected). The black bar represents the TRPV-1 agonist capsaicin mediated dye uptake in untreated control cells. This dye uptake is significantly inhibited by MINO (grey bar) and TRPV-1 antagonist capsazepine (CPZ, grey patterned bar). The error bars represent S.E.M, * $P<0.05$ calculated by one-way ANOVA (Dunnett’s post-test).
3.5 Key Points

- MINO inhibits P2X7 and P2X4 mediated dye uptake and Ca\(^{2+}\) influx at higher concentrations.
- MINO has an inhibitory effect on P2X7 mediated IL-1\(\beta\) secretion and cell proliferation.
- There is no difference in the inhibitory effect of MINO among the different species (rat and humans) of P2X7.
- The P2X7 and P2X4 mediated responses are reduced but not completely blocked by the drugs.
- Pre-incubation of P2X7 and P2X4 expressing cells with MINO was required for the inhibition of responses.
- Among other tetracycline derivatives tested, DOX has similar efficacy as MINO.
- Rat TRPV-1 mediated dye uptake is inhibited by MINO.

3.6 Discussion

This study provides evidence in support of the inhibitory action of MINO on P2X mediated responses. Membrane pore formation is a characteristic feature of P2X7 receptors, but a similar increase in membrane permeability is also displayed by the structurally homologous P2X4 receptors. The formation of membrane pores was monitored by stimulating the BV-2 microglial cell line with ATP and then the dye uptake was measured in these cells in the presence and absence of MINO. A significant inhibition in the dye uptake was observed in the presence of MINO (Figure 3.3 and Figure 3.4). These results indicate that MINO may be acting on P2X7 and P2X4 receptors which are expressed endogenously in microglia.

The activation of P2X and P2Y receptors is known to cause the entry of extracellular Ca\(^{2+}\) into the cells (325). Microglia are electrically non-excitable cells which maintain their Ca\(^{2+}\) homeostasis primarily by Ca\(^{2+}\) release from intracellular stores and with Ca\(^{2+}\) entry through
plasma membrane via ligand gated and store operated ion channels (118, 326). Stimulation of microglial cells with low concentration of (< 100 µM) ATP activates the P2X4-mediated Ca^{2+} entry while activation by high concentration of ATP (1 mM) activates P2X7-mediated Ca^{2+} influx as well. The P2X4 receptors have higher affinity and the Ca^{2+} entry response mediated by these receptors is quick, large and reversible whereas activation with higher ATP causes an irreversible sustained Ca^{2+} response (probably via P2X7 mediated membrane pore formation). The sustained change in Ca^{2+} was measured by using the Fura-2 AM dye and it was observed that MINO blocked the increase in intracellular Ca^{2+} associated with purinergic receptor activation (Figure 3.5). This increase in Ca^{2+} is essential, although not adequate, for activation of the cytotoxic and inflammatory actions of microglia (327). The P2X7 antagonist AZ10606120 was able to abolish the dye uptake and Ca^{2+} influx in these assays, supporting our hypothesis that MINO is targeting P2X7 to inhibit microglial activation. MINO also inhibited the Ca^{2+} influx mediated by different subtypes of metabotropic P2Y receptors expressed endogenously in HEK-293 cells. Activation of P2Y receptors leads to increase in cytosolic Ca^{2+} levels via the phosphoinositol turnover dependent Ca^{2+} release from intracellular organelles (328). The inhibitory effect of MINO on P2Y-mediated Ca^{2+} entry indicates that there may be a common signalling pathway mediated by purinergic receptor system in microglia which is being targeted by MINO (Figure 3.20).

The involvement of P2X7 receptors in activation of inflammatory pathways in microglia is well established (303). It is known that P2X7 activation induces a range of downstream signalling events including elevation of intracellular Ca^{2+} to mitochondrial effects, caspase activation, inflammasome activation, ROS production, PLA_2 and PLD activation, MMP9 activation, NF-κB activation and microglial proliferation (136, 178). The reduction in IL-1β release and cell proliferation in the present study provide a reliable evidence for the inhibitory effect of MINO in P2X associated downstream signalling in microglia. There appears to be
substantial overlap between signalling pathways activated by P2X7 and the signalling pathways blocked by MINO. Moreover, there is a high similarity in the diseases where P2X7 is thought to play a role in pathogenesis, for example rheumatoid arthritis, ischaemic stroke, depression, pain (136, 329, 330) and diseases where MINO has a proven beneficial effect.

After establishing the inhibitory effect of MINO on P2X7 responses in BV-2 microglia, the direct effect of the drug on P2X7 and P2X4 was established by over-expressing these receptors in HEK-293 cells. The HEK-293 cells expressing these receptors provide a good model for testing the effect of drug on each receptor type in isolation. MINO inhibited the dye uptake in both P2X7 and P2X4 expressing cells in a non-competitive, concentration-dependent manner (Figures 3.9 to Figure 3.13).

The inhibitory action of MINO requires -incubation with the drugs for 15-30 minutes prior to application of the agonist. Prolonged exposure of receptor to drugs may sometimes lead to the internalisation of the receptor, causing downregulation of the associated responses. The data from immunofluorescence study indicates that there was no difference in the surface expression of P2X7 in cell with MINO pre-treatment. The possibility of interference of MINO with fluorescence intensity was ruled out by addition of MINO after YOPRO-1 uptake. No reduction in fluorescence confirmed that inhibitory effect of the drug is due to its effect on the receptor and not because it quenches the fluorescence (Figure 3.16 and Figure 3.17). Also, 0.01 % Triton X-100 was used to lyse the cell membrane in order to obtain maximum dye uptake. HEK-P2X7 cells pre-incubated with MINO did not show inhibition in dye uptake providing evidence that MINO acts via P2X mediated responses and not on fluorescence of the dye itself (Figure 3.15).

In addition to P2X receptors, other receptors such as transient receptors, TRPV-1 and TRPA-1 have been reported to show increase in membrane permeability on activation by their ligand (331-333). MINO has been reported to inhibit the capsaicin-induced membrane pore in TRPV-1 expressing cells. These results were confirmed in the present study in which MINO reduced
the rat TRPV-1 mediated YOPRO-1 uptake (Figure 3.21). These results suggest that MINO might be acting on a common pore dilation mechanism.

To further validate the results obtained with cell lines, MINO and DOX were tested in primary peritoneal macrophages, which express P2X and P2Y receptor similar to microglia. The data from these experiments were consistent with our data from previous experiments with BV-2 microglia and the HEK-293 cell system.

Taken together, the results from the above study suggest that MINO may be inhibiting the formation of macropores by P2X receptors in microglia. The process of macropore formation has been extensively studied for P2X7 receptors, but also occurs in response to activation of P2X4 receptors in microglia (315).

An effect of MINO on the long term, pro-inflammatory functions of P2X receptors, rather than the short-term signalling functions is consistent with the relative lack of side effects of this drug when given systemically. For instance drugs blocking the P2X4 receptor may have cardiac side effects due to the signalling role of P2X receptors in the heart (334). Thus, compounds which block pore formation function of P2X receptors may be useful in therapeutic applications where MINO has shown promise.

MINO is known to play a role in attenuating the symptoms of a variety of pathological conditions and a number of mechanisms have been proposed to explain its mode of action. However, despite the mounting need for development of better drugs with similar efficacy, the direct molecular targets of MINO that mediate its in vivo actions still remain elusive. In the present study, we propose a novel target of MINO’s inhibitory action on microglia, the membrane pore forming purinergic receptors, P2X7 and P2X4.

In the study, we also tested tetracycline (TET) and its analogues doxycycline (DOX) and tigecycline (TIGE) in order to understand the structure-function relationship of the drug
towards the receptor (Figure 3.17). While DOX mimicked MINO its efficacy in inhibiting P2X mediated macropore formation, parent compound TET and the analogue TIGE had no inhibitory effect at all. MINO and DOX have a polyphenolic vitamin E like structure with a number of different substituent groups (335). The presence of dimethylamino group on C-7 is suggested to improve the steric hindrance of these compounds (254). This structural similarity between MINO and DOX could be a major contributor to their receptor binding and their superior inhibitory action on P2X7 and P2X4 responses.

The concentrations of both MINO and DOX required for inhibiting P2X responses are in the high micromolar range. However, many of the in vitro studies using MINO also report the use of micromolar concentrations (336, 337). Such concentrations are likely achievable in vivo, with typical serum concentrations in humans reaching > 0.0087 µM (338). In addition, both MINO and DOX are highly lipophilic and can cross the blood-brain barrier; therefore, CSF concentrations may be higher than serum concentrations. The higher concentrations of these drugs in brain can explain their capacity to activate purinergic receptors in microglia. The mean levels of MINO in the brain have been reported to be three fold more than DOX (339). There are some common side effects related to long term MINO uptake which include nausea, dizziness, hyperpigmentation, and symptoms of systemic lupus erythematosus (340). It is observed that in most cases the symptoms disappear once the drug intake is discontinued. Due to these reasons, the British National Formulary instructs a follow up after every 3 months in case long term MINO treatment is administered to a patient. Even though MINO is well accepted by most patients, current therapy is moving towards using DOX as a substitute for MINO as it has similar efficacy but reduced incidence of side effects such as nausea, hyperpigmentations and hypertoxicity often associated with MINO treatment (254). Typically, MINO and DOX are thought to be safe drugs with limited side effects but one of the goals of understanding how these second-generation tetracyclines exert their beneficial anti-
inflammatory and neuroprotective actions is that better, high affinity drugs can be developed. The results from our study suggest that antagonising P2X receptors may have a similar effect as MINO and this idea certainly warrants further investigation.

**Mechanism of action of MINO via P2X receptors**

To witness the inhibitory response of MINO, exposure of cells to the drug prior to receptor activation is essential. This observation leads us to the question whether MINO acts directly on the receptor channel protein or not. To understand whether MINO affects the P2X mediated ion channel activity or the membrane pore formation; we have to first consider the mechanism underlying the membrane pore formation. P2X receptors are trimeric protein complexes with intrinsic ion channel activity. Each constituent subunit has two transmembrane domains and there are three ATP binding pockets on the interface of each of these subunits. On ATP binding to the binding pockets, the extracellular domain of the receptor undergoes a conformational change, making way for the small cations to pass through. Ser342 and Leu351 provide the physical gate of the ion permeation pathway in the crystal structure of rat and human P2X7 (341). Extensive studies have been conducted in order to elucidate the exact mechanism of pore formation but no unified mechanism has so far been accepted (149, 152, 342-344). However, two different mechanisms have gained general popularity. According to the first one, membrane pore formation is an intrinsic property of the ion channel and longer agonist binding leads to dilation of the small permeation pathway (141). The second hypothesis suggests the involvement of another class of membrane proteins such as pannexin-1 (343, 344). There are studies that have provided data for or against each of these hypotheses. The biophysical approach for studying these mechanisms involves the use of N-methyl D-glucosamine (NMDG⁺) for measuring P2X receptor currents using whole cell patching (345). The whole-cell patch recordings from this method show that there is an increase in NMDG⁺ permeability as the time of receptor activation increases (346). In the present study, confirmation was not
obtained to support the effect of MINO on channel current by measuring patch clamping. In the FluxIon automated patch clamp experiments attempted, the pre-incubation of cells with MINO for 15-30 minutes prior to running measuring current, did not result in robust response (probably due to failure of giga-seal formation). This pre-incubation did not affect any other assays as the cells in all those cases were sticking to the poly-L lysine coated plate surface.

The second experimental method popularly used for measurement of P2X dependent large pore formation is the measurement of agonist induced intracellular accumulation of fluorescence dyes such as ethidium and YOPRO-1. This technique was employed in the present study to measure P2X7 pore formation and the effect of tetracyclines on that activation. Biophysical studies conducted on the basis of the crystal structure of P2X4 (elucidated by Hattori and Gouaux in 2012) have established that the physical gate constituting Ca atoms of three Ser342 residues is 6.4 Å from the central axis (137). YOPRO-1 has a molecular size of 7 Å x 8 Å x 19 Å (347). Thus, either the channel has to dilate as wide as 14 Å or there has to be formation of pores by P2X7 receptors. Pore formation via P2X4 receptors in microglia also appears to be independent of other pore forming proteins such as pannexins (315). In the present study, a linear increase in dye uptake was observed for a period of 5 minutes and this dye uptake was seen to be reduced in cells pre-exposed to MINO. This observation ascertains that MINO has an effect on increased membrane permeability associated with prolonged P2X activation. This could suggest direct action on the channel, since ATP responses in these cells are not pannexin dependent, even when pannexin proteins are co-expressed (348).

While it is possible that MINO is acting on some other, yet to be identified protein, such a protein would need to be expressed in HEK-293 cells and to have a similar role in the function of both P2X4 receptors and P2X7 receptors. While the sustained ATP-induced Ca\(^{2+}\) increase is initiated by P2X receptors, other mechanisms may contribute, and these are also potential targets of MINO. Store operated Ca\(^{2+}\) entry (SOCE) does contribute to the initial microglial
responses to ATP (349), although its role in the sustained Ca\(^{2+}\) responses to prolonged P2X activation have not been fully investigated and there is evidence that SOCE in microglia is not inhibited by MINO (350).

MINO is reported to act directly on several molecular targets implicated in mediating its anti-inflammatory effects (351) including PARP-1 and MMPs, but definitive links between these targets and functional inhibition, particularly in vivo, have yet to be established (289, 352). The anti-apoptotic action of MINO is perhaps the exception, having been linked to its direct inhibition of the mitochondrial membrane pores that lead to release of cytochrome c (297). It is tempting to speculate that the inhibition of pore formation by MINO is a mechanism common to its anti-inflammatory and anti-apoptotic actions. While many selective P2X7 antagonists have already been developed and tested in pre-clinical studies (136), specific compounds for P2X4 are limited. However, the use of a non-selective purinergic receptor antagonist, suramin, has recently been demonstrated to have a significant effect in models of autism and fragile X syndrome (353, 354) suggesting that compounds blocking multiple purinergic receptors can be advantageous.

3.7 Conclusion

Taken together, the data from this chapter suggests that MINO and DOX inhibit purinergic receptor mediated responses in microglia. In line with the studies suggesting that the inhibitory effect of MINO and related drugs on microglia-mediated neuroinflammation may be due to its effect on multiple targets; we confirm that the modulation of purinergic responses is one of the mechanisms involved. This study was designed to investigate the possibility of purinergic receptors being the direct targets of MINO and our results suggest that there may be an action of the drug on P2X7 and P2X4 receptors. Whether this is the only mechanism involved in the inhibitory effect of MINO on microglial proliferation is not conclusive.
CHAPTER 4
PURINERGIC RECEPTOR MODULATION BY GINSENOSESIDES

4.1 Introduction

Panax ginseng vern: ginseng (C.A. Mayers) is a Chinese herb which is widely used in traditional medicine as an immune tonic believed to enhance physical strength, resistance and longevity (355). The word Panax is derived from “panacea” which means a solution or remedy for all difficulties or diseases. This botanical nomenclature is an indicator of the range of demonstrated pharmacological effects of this plant in alternative medicine. Traditionally, ginseng is used either in a freshly harvested form referred to as “fresh ginseng” or as long storage dried forms called “white” and “red” ginseng. Around 200 chemical components from ginseng have been isolated and identified so far (356). The main bioactive components among these are ginseng saponins, also known as ginseng glycosides or ginsenosides. These ginsenosides act as a beneficial defence mechanism for the plants because of their antimicrobial/antifungal properties and their bitter taste makes the plants non-palatable for grazing animals (357).

Figure 4.1 Chinese herb Panax ginseng has a number of reported medicinal applications
(A) Fresh P. ginseng plant with roots (B) Dried roots
Ginsenosides are said to be efficacious in attenuating the clinical symptoms of many diseases such as cancer, inflammation, diabetes, ischemia, stroke, neurodegeneration and cardiovascular disorders (16, 358, 359). Due to its potential in chemoprotection or adjuvant treatment, ginseng is one of the most widely purchased herbs in the United States and European countries (360). Generally, ginsenosides are named as ‘Rₓ’ where R stands for root and x describes the chromatographic polarity in alphabetic order. Thus, ginsenosides Ra is most polar and Rh has least polarity among the ginsenosides isolated and characterised (361). Structurally, each ginsenoside is made up of a four-ring steroid structure, each with distinct carbohydrate moieties attached to it. Depending on the positions of carbohydrate moieties attached to the C-3 and C-6 carbon atoms of the steroid rings and their aliphatic side chains, the ginsenosides have been classified as protopanaxadiols (PPDs) with two positions for attachments or protopanaxatriols (PPTs) with positions for three attachments. Oleanolic ginsenosides and ginsenosides metabolites make up a third class of compounds (362). The pseudoginsenoside F11 belongs to the PPT class even though there is a tetrahydrofuran ring instead of a carbon chain at position 20. Some rare ginsenosides with oleanolic acid type structure and octillool type structure are also been identified (363). Table 4.1 illustrates the classification of various ginsenosides based upon their backbone structure. The substituent groups of some of the well-known PPD and PPT based ginsenosides in Table 4.2.

The composition and type of bioactive constituent present in the plant depends on number of factors including species, age of the plant, the plant part used, cultivation, harvesting and extraction methods (364). In general, aging increases the saponin content of the plant. Air-drying the ginseng roots yields white ginseng, which is known to have lower saponin content than other forms. Red ginseng is processed by boiling at 100 °C before drying (365). The heat transformation and deglycosylation is thought to be an explanation for the higher saponin profile. Successful efforts have been made to improve the bioactive component content of ginseng by steaming the roots at 120 °C. This type of ginseng is referred to as sun ginseng. It
contains more ginsenosides with reported anti-tumour properties than the other types of ginseng (366).

To maintain consistency among various pharmaceutical preparations, many standardised ginseng formulations are commercially available. Two of the more commonly used formulations are G115 and NAGE (367). G115 is manufactured by the pharmaceutical company Pharmaton SA (Switzerland) using the root of *P. ginseng* with the total ginsenoside content adjusted to 4 % (higher Rg1). NAGE is a root extract of *P. quadrifolius* with a ginsenoside content of 10 % (high Rb1, Re content) and is manufactured by Canadian Phytopharmaceuticals Corporation (Canada) (361, 367). The defined ginsenoside content of commercially available extracts is important in pre-clinical testing and clinical trials.

Most of the natural herbs are consumed orally and are subjected to degradation in the gastrointestinal tract. The health benefits attributed to a whole plant extract can be credited only to a few metabolites that are effectively absorbed through the intestines after biodegradation in the gut. In order to gain a better understanding of the effects of ginsenosides in humans, the pharmacological properties of bioactive components and metabolites need to be studied in detail.
Table 4.1 Classification of ginsenosides based upon their backbone structures [adapted from Chen et al. 2009 (368)]

<table>
<thead>
<tr>
<th>Class</th>
<th>Backbone type</th>
<th>Structure</th>
<th>Representative ginsenosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protopanaxadiols</td>
<td>Danmarane</td>
<td><img src="image1" alt="Danmarane Structure" /></td>
<td>Rb1, Rb2, Rg3, Rh2, Rh3</td>
</tr>
<tr>
<td>Protopanaxatriols</td>
<td>Additional hydroxyl group on C6 in a dammarane structure</td>
<td><img src="image2" alt="Additional Hydroxyl Structure" /></td>
<td>Rg1, Rg2, Rh1</td>
</tr>
<tr>
<td>Ocotillol type</td>
<td>Five membered epoxy ring at C20</td>
<td><img src="image3" alt="Five Membered Structure" /></td>
<td>F11</td>
</tr>
<tr>
<td>Oleanolic acid type</td>
<td>Pantacyclic triterpenoid base</td>
<td><img src="image4" alt="Pantacyclic Structure" /></td>
<td>Ro</td>
</tr>
</tbody>
</table>
Table 4.2: Substituent R groups of some of the common ginsenosides [adapted from Sergiy Oliynk and Seikwan oh, 2013 (369)]

<table>
<thead>
<tr>
<th>Protopanaxadiols</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginsenoside</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb1</td>
<td>Glc-Glc</td>
<td>H</td>
<td>Glc-Glc</td>
</tr>
<tr>
<td>Rb2</td>
<td>Glc-Glc</td>
<td>H</td>
<td>Glc-ara(p)</td>
</tr>
<tr>
<td>Rc</td>
<td>Glc-Glc</td>
<td>H</td>
<td>Glc-ara(f)</td>
</tr>
<tr>
<td>Rd</td>
<td>Glc-Glc</td>
<td>H</td>
<td>Glc</td>
</tr>
<tr>
<td>Rg3</td>
<td>Glc₂ₙ₋₁</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Rh2</td>
<td>Glc</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Gp-XVII (M9)</td>
<td>Glc</td>
<td>H</td>
<td>Glc₂ₙ₋₁Arab</td>
</tr>
<tr>
<td>Mb(M7)</td>
<td>Glc</td>
<td>H</td>
<td>Glc₂ₙ₋₁Arab</td>
</tr>
<tr>
<td>M6</td>
<td>Glc</td>
<td>H</td>
<td>Glc₂ₙ₋₁Arab</td>
</tr>
<tr>
<td>Gp-LXXV (M13)</td>
<td>H</td>
<td>H</td>
<td>Glc₂ₙ₋₁Glc</td>
</tr>
<tr>
<td>F2</td>
<td>Glc</td>
<td>H</td>
<td>Glc</td>
</tr>
<tr>
<td>Mc</td>
<td>H</td>
<td>H</td>
<td>Glc₂ₙ₋₁Arab</td>
</tr>
<tr>
<td>C-Y</td>
<td>H</td>
<td>H</td>
<td>Glc₂ₙ₋₁Arab</td>
</tr>
<tr>
<td>Compound K</td>
<td>H</td>
<td>H</td>
<td>Glc</td>
</tr>
<tr>
<td>Panaxadiol (PPD)</td>
<td>h</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protopanaxatriols</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginsenoside</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Re</td>
<td>H</td>
<td>-O-Glc-Rha</td>
<td>Glc</td>
</tr>
<tr>
<td>Rf</td>
<td>H</td>
<td>-O-Glc-Glc</td>
<td>H</td>
</tr>
<tr>
<td>Rg₁</td>
<td>H</td>
<td>-O-Glc</td>
<td>Glc</td>
</tr>
<tr>
<td>Rg₂</td>
<td>H</td>
<td>-O-Glc-Rha</td>
<td>H</td>
</tr>
<tr>
<td>Rh₁</td>
<td>H</td>
<td>-O-Glc</td>
<td>H</td>
</tr>
<tr>
<td>Panaxatriol (PPT)</td>
<td>H</td>
<td>-OH</td>
<td>H</td>
</tr>
</tbody>
</table>

Where Glc=Glucose; Araf= arabinofuranose; Arabarabinopyranose; Rha = rhamanopyrinoside
4.2 Biodegradation and bioavailability of ginsenosides

Owing to the hydrophilic nature of the bioactive constituents of ginseng extract, not all of these are easily absorbed from the intestines following oral ingestion. On consumption of ginseng extract, the large molecular weight ginsenosides are encountered by intestinal flora and are metabolized into different chemical forms by the removal of carbohydrate groups (370). Compound K (CK) and PPD are the major products following microbial degradation of the PPD class of ginsenosides within the gut while PPT gets further degraded to F11 (Table 4.3). Absorption of the microbiologically transformed ginseng metabolites is relatively easier partly because of their non-polar nature (361). The absorbed metabolites may be responsible for the pharmacological properties associated with ginseng extracts.

![Figure 4.2 Possible biotransformation of ginsenosides by lactic acid bacteria in human intestines (371)](image-url)
The transport of ginsenosides across the intestinal mucosa is an energy dependent and non-saturable process. The bioavailability of ginsenosides and their metabolites from intestines is not too high but efforts are being made to find methods of increasing this. Among the strategies applied for this purpose is dissolving the ginsenoside complex in medium chain fatty acids to make a lipid preparation (372), adrenalin-ginsenoside co-administration (373), p-glycoprotein efflux suppression (374).

Compound K (CK) is the major metabolite present in the bloodstream after oral administration of ginseng extracts with large molecular weight protopanaxadiol ginsenosides Rb1, Rb2, Rc and Rd. Plasma concentrations of CK have been found to be around 70 ng/ml in humans (375). CK is well known for its anti-cancer, anti-inflammatory and anti-oxidant affects (376-378) and the pharmacokinetics of this active metabolite are well studied. After 36 hours ingestion, the mean maximum plasma concentration of CK is found to be significantly higher than parent compound Rb1. It has also been observed that the absorption of CK is greater in females than males and is also accelerated in individuals taking a high fat diet (379). CK is known to be more bioavailable than other ginsenosides and is related to a plethora of potential health benefits (380). A deeper understanding of the mechanism of action of CK would determine the precise molecular targets of the compound, which would aid in obtaining maximum benefits of this herb in treatment of various disorders.

4.3 Mechanism of ginsenosides action

Ginsenosides are well known for their anti-oxidant action (381-385), their effect on the eNOS system (386-390), their ability to interact with ion channels and membrane receptors along with the associated signalling pathways (391-395). It is believed that molecular targets of ginsenosides are either on the cell membrane or inside the cells, depending upon the hydrophobicity of the ginsenosides (361).
Even though the pharmacological effect of these bioactive saponins are well described, their precise molecular targets are not well established. Of foremost interest is the effect of ginsenosides on voltage gated and ligand gated ion channels. Inhibition of Ca\(^{2+}\) influx is thought to prevent Ca\(^{2+}\) overload and ROS production (396). This has been proposed as a potential mechanism of protective effect of ginsenosides towards oxidative damage. For example, in ventricular myocardiocytes, Rb1, Rb2, Rb3 and Rc were found to decrease the inhibit Ca\(^{2+}\) influx and lowering ROS production in these cells (397). Similarly, ginsenoside Rg1 elicited a neuroprotective effect in ischemia-hypoxic conditions by inhibiting Ca\(^{2+}\) influx through voltage gated Ca\(^{2+}\) channels and NMDA receptors (296). Ginsenosides are known to be smooth muscle and vascular endothelium relaxants. PPD was observed to cause vasorelaxation in rat aortic rings through the activation of Ca\(^{2+}\) activated K\(^{+}\) channels, inhibition of voltage-gated and receptor associated Ca\(^{2+}\) channels (398). Ca\(^{2+}\) activated K\(^{+}\) channels (K\(_{Ca}\)) in coronary arteries are associated with production of nitric oxide (NO), a vasodilator. It is suggested that potentiation of K\(_{Ca}\) channels may have the potential of improving endothelial cell function in cardiovascular disease (399). Ginsenoside Re was found to increase the K\(_{Ca}\) activity in Human Coronary Artery Endothelial Cells (HCAEC) thereby providing a plausible explanation to the beneficial effects of ginseng on heart functions (400). Rg3 was found to inhibit voltage gated Ca\(^{2+}\), K\(^{+}\) and Na\(^{+}\) channels (401). Thus, it appears that ginsenosides may decrease the influx of positively charges ions and enhance the efflux of negative ions to reduce the excitability of excitable cells like neurons and muscles. Additionally, it has been suggested that the regulation of ion channels by ginsenosides may be through action on the extracellular side (402).

Ginsenosides have a structural similarity with steroids; therefore, it has been suggested that some of their activities might be through non-genomic pathways from inside the cells (403). It is further speculated that length of treatment may be a determining factor in the ginsenoside
action. Long term (more than 10 minutes) treatment with ginsenosides may allow them time to enter cells across the plasma membrane and act via steroid receptors as well as cause changes in cell proliferation pathways. Short term treatment of 1-2 minutes may just be enough for these hydrophilic molecules to interact with plasma membrane ion channels/receptors to cause changes in membrane polarization and downstream signalling (404).

In 2013, Im and Nah reviewed ginsenoside pharmacology with a specific emphasis on contrasting the effects of ginsenosides and gintonin on ion channels (391). Initial experiments performed with ginseng extract found a potentiating effect on Ca\(^{2+}\) signalling. Individual ginsenosides when tested were unable to mimic this response as these were observed to stabilize the membrane potential by inhibiting Ca\(^{2+}\) influx. Gintonin, a glycosylated protein complex containing lysophosphatidic acid, on the other hand was found to have high affinity for Ca\(^{2+}\) and caused potentiation of ion channels. Im & Nah concluded that ginsenosides and gintonin may be acting as two complementary and opposite factors of ginseng pharmacology (391). The authors proposed that the anti-cancer and pro-apoptotic properties of ginseng may be attributed to its gintonin component. Gintonin, even though it causes an increase in Ca\(^{2+}\) influx, is present in very low levels in ginseng extract (405). These observations highlight the importance of understanding the actions of ginsenosides and other bioactive compounds found in ginseng both in isolation and in combination. Only then will we be in a position to account for the overall health benefits or side effects of this herb.
Table 4.3 Recent studies correlating the physiological effects of ginsenosides have been correlated with their capability to modulate various signalling pathways.

<table>
<thead>
<tr>
<th>Ginsenosides</th>
<th>Target signalling pathway</th>
<th>Physiological effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD</td>
<td>PI3K/Akt/ERK &amp; Raf/MEK/ERK</td>
<td>Enhanced angiogenesis</td>
<td>(406)</td>
</tr>
<tr>
<td>Re</td>
<td>PPAR-γ signalling</td>
<td>Gap junction remodelling</td>
<td>(407)</td>
</tr>
<tr>
<td>Rb3</td>
<td>Endothelial to mesenchymal transition (EndoMT)</td>
<td>Anti-fibrosis</td>
<td>(408)</td>
</tr>
<tr>
<td>Rg3</td>
<td>AMP-activated protein kinase</td>
<td>Mitochondrial protecting, autophagy enhancing role</td>
<td>(409)</td>
</tr>
<tr>
<td>Rg5</td>
<td>Inflammatory and apoptotic pathways</td>
<td>Hepatoprotective Anti-apoptotic</td>
<td>(410)</td>
</tr>
<tr>
<td>Rh2</td>
<td>Orphan Nuclear Receptor Nur77</td>
<td>Pro-apoptotic(anti-cancer)</td>
<td>(410)</td>
</tr>
<tr>
<td>Re</td>
<td>PI 3-K/Akt Signalling</td>
<td>Neuroprotective</td>
<td>(411)</td>
</tr>
<tr>
<td>Rg1</td>
<td>Akt-FoxO1 Interaction</td>
<td>Gluconeogenesis-lowering effect(anti-type II Diabetes)</td>
<td>(363)</td>
</tr>
<tr>
<td>Rg3 derivative HRG</td>
<td>VEGF, b-FGF, MMPs</td>
<td>anti-angiogenic effect</td>
<td>(412)</td>
</tr>
<tr>
<td>Rg3</td>
<td>Eicosanoids</td>
<td>Anti-inflammatory</td>
<td>(413)</td>
</tr>
<tr>
<td>Rg3</td>
<td>STAT5-PPARgamma</td>
<td>Anti-obesity</td>
<td>(414)</td>
</tr>
<tr>
<td>Rd, Re, FloralGA</td>
<td>Melanin biosynthesis</td>
<td>Melanogenesis inhibitor</td>
<td>(415)</td>
</tr>
<tr>
<td>Rh2</td>
<td>Lytic replication of human gamma herpes virus</td>
<td>Anti-viral</td>
<td>(416)</td>
</tr>
<tr>
<td>Rg3</td>
<td>Microglial activation ,NF-κβ pathway</td>
<td>Anti-depressant Anti-inflammatory</td>
<td>(417)</td>
</tr>
<tr>
<td>Rg1</td>
<td>Vaccine adjuvant</td>
<td>Anti-cancer</td>
<td>(418)</td>
</tr>
<tr>
<td>Rb3,Rd</td>
<td>iNOS, STAT3/pSTAT3, Src/pSrc</td>
<td>Anti-cancer Pro-biotic effect</td>
<td>(419)</td>
</tr>
<tr>
<td>Rg3</td>
<td>NF-κβ/ p38MAPK pathway</td>
<td>Anti-oxidant and anti-senescence in astrocytes</td>
<td>(420)</td>
</tr>
<tr>
<td>Rd, Re co-treatment</td>
<td>Apoptotic signalling, ROS generation</td>
<td>Anti-oxidant, neuroprotective</td>
<td>(421)</td>
</tr>
<tr>
<td>Rg3</td>
<td>phosphoinositide 3-kinase/Akt pathway</td>
<td>Anti-apoptosis</td>
<td>(422)</td>
</tr>
</tbody>
</table>
As discussed in the previous chapters, P2X receptors are trimeric, ATP-gated cation channels that are permeable to Na\(^+\), K\(^+\) and Ca\(^{2+}\) ions (423). The seven subtypes of the P2X receptor family have a widespread distribution and are implicated in a number of physiological processes such as synaptic transmission, nociception, inflammation and taste sensation (306). P2X4 and P2X2 are known to interact with extracellular ATP to form a large conductance pore on the cell membrane, similar to closely related P2X7 (136). Upon receptor activation, the membrane pore change conformation which causes large cation flux across the cell membrane, resulting in membrane depolarization and activation of a number of signalling pathways related to physiological processes such as pain and inflammation (424). Involvement of purinergic receptors in the initiation and progression of inflammatory processes is well documented with P2X7 being said to have a more prominent role than structurally homologous P2X4 (185). It has also been suggested that in activation of the inflammasome, P2X4 may act as an initial trigger while P2X7 in combination with pannexin-1 may amplify the signal (184). More recently, studies conducted to understand the role of P2X4 in inflammation suggest that this ion channel may have a greater role to play in inflammatory processes than previously thought. Inhibition of P2X4 in a collagen-induced model of arthritis can affect the development of arthritis and autoimmunity in mice, indicating a direct involvement of P2X4 in synovial inflammation and joint destruction (425). ATP-P2X4R signalling is implicated in airway inflammation and airway re-modelling in allergic asthma in mice (426). P2X4 is also involved in release of an early inflammatory mediator prostaglandin E (PGE\(_2\)) (427). P2X4 deficiency in mice leads to impaired inflammasome activation, lack of pain hypersensitivity and complete absence of PGE\(_2\) in tissue exudates of these animals (428). There is also evidence that P2X2 antagonism can cause relief from pain-related responses and urinary inconstistence (429).
All these and more studies provide evidence in support of the view that P2X receptors are key modulators in inflammatory pathways and finding more pharmacological tools to target these receptors in order to manipulate the physiological processes they control remain a priority.

4.4 Research Question

In this study, we investigated the effects of ginseng extract and individual purified chemicals on ATP-gated P2X7 receptors in our quest to find novel pharmacological modulators for this receptor. Once the activity was established, the specificity of these compounds on other P2X and P2Y receptors was then examined.
4.5 Results

4.4.1 Effect of ginseng extract G115 on human P2X7

To establish any potential effects of ginseng extract on P2X7 receptors, the YOPRO-1 iodide dye uptake assay was utilised. For initial screening of compounds an approximate EC₅₀ concentration of ATP (200 μM) was used in order to see either potentiation or inhibition of the response. HEK 293 cells expressing human P2X7 (stable expression) were used. A standard formulation of ginseng known as G115 was tested as it is widely used as an active ingredient in modern medicine (430). Pre-treatment of HEK-hP2X7 cells for 10 minutes with 100 μg/ml G115 dissolved in water increased the rate of ATP-induced dye uptake by around two-fold (Figure 4.3). G115 without ATP did not induce dye uptake in HEK-hP2X7 cells, suggesting this was not a non-specific effect on the cells (Figure 4.3).

G115 contains a fixed amount (4 % w/w) of eight ginsenosides from both PPD and PPT chemical classes, namely Rb1, Rc, Rd, Re (PPD ginsenosides) and Rb2, Rf, Rg1, Rg2 (PPT ginsenosides) (431). After confirming the effect of G115 on P2X7-mediated dye uptake, it was important to establish whether the observed effects with G115 resulted from one or more specific ginsenosides(s) in the formulation. Therefore, 14 purified ginsenosides were tested in a screening assay encompassing the eight ginsenosides in G115 plus the principal intestinal metabolites, Rh1, Rh2, Rg3, Compound K (CK), and the two aglycones, PPD and PPT. All ginsenosides (10 μM) were pre-incubated with the cells before the addition of ATP (200 μM). None of the ginsenosides stimulated dye uptake on their own, however, four PPD ginsenosides, Rb1, Rd, Rh2 and CK, significantly increased the rate of dye uptake after stimulation by ATP (Figure 4.4). In contrast, ginsenosides of the PPT series had no significant effect on the ATP-induced dye uptake (Figure 4.2).
4.4.2 Potentiation of human P2X7 mediated dye uptake by the ginseng metabolite CK

As CK was found to be the most effective among the ginsenosides tested in the above assay, the investigation focused on this ginsenoside to further examine the potentiating action on P2X7 receptors in more detail. To determine if CK was acting as a positive allosteric modulator of P2X7 receptors, a full concentration-response curve for ATP in the absence and presence of 10 μM CK was performed (Figure 4.5). Potentiation by CK caused a leftward-shift of the concentration-response curve reducing the EC\text{50} for ATP from 259.6 μM (95 % confidence interval – 228.8 to 294.5) to 80.7 μM (95 % confidence interval – 69.5 to 94), increased the maximum ATP response and required a threshold concentration of ATP (∼ 50 μM) (Figure 4.3). In contrast, when BzATP was used as a full agonist at human P2X7 receptors (234), CK decreased the EC\text{50} value from 20.3 μM (95 % confidence interval – 16 to 25.7) to 5.9 μM (95% confidence interval – 4.6 to 7.6) (Figure 4.5). The leftward shifts of the dose response curves in the presence of both ATP and BzATP were statistically significant (P < 0.01). The response induced by ATP and CK was solely dependent on P2X7 receptors as it could be completely abolished by pre-treatment with the selective P2X7 receptor antagonist AZ10606120 (10 μM) (Figure 4.5). Similar to its metabolite CK, the parent compound Rd was also observed to increase the YOPRO-1 uptake via ATP-activated P2X7. Various concentrations of Rd were tested on HEK 293 cells stably expressing hP2X7 and it was found that Rd increased the dye uptake in these cells in concentration-dependent manner (Figure 4.6).

4.4.3 Potentiation of human P2X7 mediated Ca\textsuperscript{2+} influx by ginseng metabolite CK

Activation of P2X7 receptors can result in a sustained rise in intracellular Ca\textsuperscript{2+} leading to either proliferation/activation or cell death, depending on the magnitude and extent of channel activation (202). Therefore, it was important to establish whether the ginsenoside potentiation of P2X7 receptor responses observed in dye uptake experiments led to physiologically relevant
sustained increases in intracellular Ca\(^{2+}\) concentration. Intracellular Ca\(^{2+}\) responses were measured in HEK-hP2X7 cells using the ratiometric Ca\(^{2+}\) indicator dye Fura-2 AM. An initial transient rise in intracellular Ca\(^{2+}\) following ATP addition was observed, which is attributed to the activation of G-protein coupled P2Y receptors. However, an additional sustained elevation in intracellular Ca\(^{2+}\) on addition of ATP concentrations > 100 μM was observed following the transient peak (Figure 4.7). This sustained elevation of intracellular Ca\(^{2+}\) is a characteristic feature of P2X7 receptor activation (136). In the presence of CK, the sustained Ca\(^{2+}\) response to 200 μM ATP was further increased (Figure 4.8). Quantification of the changes in Fura-2 AM ratio during the sustained phase of the response (150-300 seconds) showed that CK significantly increases the ATP-mediated rise in intracellular Ca\(^{2+}\) (Figure 4.8).

4.4.4 Lack of difference in effect of CK among different species of P2X7

The human, mouse and rat P2X7 cDNA have been cloned and the pharmacological properties of the corresponding proteins are well characterised (432). It has been observed that there is a significant difference in pharmacological properties of P2X7 depending upon the species of origin. For example, human and rat P2X7s have higher affinities for agonists in comparison to mouse P2X7 (432). Also, current growth and channel dilation are more rapid in human and rat P2X7 in comparison to their mouse counterpart (433). Keeping these species differences in mind, the effect of selected ginsenosides were tested on human and rat P2X7 responses using the YOPRO-1 dye uptake assay. HEK-hP2X7 and HEK-rP2X7 cells were exposed to various concentrations of ginsenosides to obtain concentration response curves. It was found that ginsenosides were able to potentiate YOPRO-1 dye uptake for both P2X7 orthologues, though (Figure 4.8). EC\(_{50}\) value for each ginsenosides for both P2X7 orthologues is listed below.
Table 4.4 Comparative list of EC$_{50}$ values of ATP for human and rat P2X7 in the presence of selected ginsenosides

<table>
<thead>
<tr>
<th>Ginsenoside</th>
<th>EC$_{50}$ human P2X7</th>
<th>EC$_{50}$ rat P2X7</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>1.21 µM</td>
<td>0.667 µM</td>
</tr>
<tr>
<td>Rd</td>
<td>0.59 µM</td>
<td>0.646 µM</td>
</tr>
<tr>
<td>Rb1</td>
<td>1.36 µM</td>
<td>1.8 µM</td>
</tr>
<tr>
<td>Rh2</td>
<td>7.85 µM</td>
<td>3.2 µM</td>
</tr>
</tbody>
</table>

4.4.5 Potentiation of P2X7 mediated cell death by ginseng metabolite CK

We next investigated whether this potentiation of ATP-induced P2X7 receptor responses could be translated to a significant downstream functional effect, such as the induction of cell death. HEK-hP2X7 treated with ATP and ginsenosides for 24 hours before MTS was added per well, 4 hours before the stipulated 24 hours’ time point. Neither 10 µM CK, DMSO nor 5 - 500 µM ATP alone induced any reduction in cell viability (Figure 4.9). In contrast, a high concentration of ATP (3 mM) for 24 h induced a significant reduction of cell viability (Figure 4.9). Treating HEK-hP2X7 with a combination of 10 µM CK and non-lethal ATP (500 µM) together, now significantly reduced cell viability (Figure 4.9). The same effect was not observed with lower concentrations of ATP. CK, therefore, seems to reduce the threshold required for the cells to commit to cell death pathways.
Figure 4.3 Standardised ginseng extract G115 increases P2X7 mediated YOPRO-1 uptake.

(A) G115 ginseng formulation and purified ginsenosides potentiate ATP-induced responses at the human P2X7 receptor. ATP-induced dye uptake was measured at 37 °C using YOPRO-1 (2 μM) as the membrane impermeant dye. Relative fluorescence units (RFU) were measured following excitation at 490 nm and emission recorded at 520 nm using a fluorescent plate reader (Flexstation 3). HEK-hP2X7 cells were pre-treated with 100 μg/ml G115 in a low divalent buffer for 10 minutes at 37 °C. ATP (200 μM) was then added to elicit a P2X7 receptor response. The mean of five individual wells was plotted. (B) Slope of the dye uptake curve data (n = 10–20 wells) was plotted for buffer control, ATP or ATP in the presence of 100 μg/ml G115. Error bars are S.E.M. * denotes P<0.05 using with Dunnett’s multiple comparison test.
Figure 4.4 Some protopanaxadiol ginseng compounds increase P2X7-mediated YOPRO-1 uptake

Fourteen purified ginsenoside compounds (protopanaxadiols in purple and protopanaxatriols in green) were tested for potentiation of P2X7 receptor responses at a concentration of 10 μM. All compounds were prepared in DMSO and were added to the low divalent assay buffer. Compounds were pre-incubated for 10 minutes prior to the addition of ATP (200 μM). The data was quantified as the slope of dye uptake curve. Error bars are S.E.M. * denotes $P<0.05$ using ANOVA with Dunnett’s multiple comparison test.
Figure 4.5 CK acts as a positive allosteric modulator of human P2X7 receptors

Dye uptake following P2X7 activation using 200 µM ATP was measured at 37 °C using YOPRO-1 (2 µM) as the membrane impermeant dye. Relative fluorescence units (RFU) were measured following excitation at 490 nm and emission recorded at 520 nm using a fluorescent plate reader (Flexstation 3). Black traces represent responses to agonist (ATP) and red traces represent responses in presence of CK + ATP (B) A concentration-response curve for the potentiating effect of CK. Concentration-response curves were generated for ATP (10 µM – 1 mM), with or without 10 µM CK. CK decreased the EC₅₀ of ATP for P2X7 activation from 259.6 µM to 80.78 µM.
Figure 4.6 CK acts as a positive allosteric modulator of human P2X7 receptors activated by BzATP.

(A) Dye uptake induced by P2X7 activation using 30 μM BzATP was measured at 37 °C using YOPRO-1 (2 μM) as the membrane impermeant dye. Relative fluorescence units (RFU) were measured following excitation at 490 nm and emission recorded at 520 nm using a fluorescent plate reader (Flexstation 3). Black open circles represent responses to agonist (BzATP) and red traces represent responses in presence of CK + BzATP. (B) Concentration-response curves were generated for BzATP (1 μM – 0.3 mM), with or without 10 μM CK. Dose response curve with varying BzATP concentrations is shown in grey while the dose response curve with ATP + CK is shown on red. CK reduces the EC$_{50}$ of BzATP for P2X7 from 20.39 µM to 5.9 µM. Non-linear dose response curves of transformed data from 3 independent experiments were fitted using GraphPad Prism.
Figure 4.7 Rd enhances the sustained dye uptake associated with hP2X7 receptor activation.

Dye uptake induced by P2X7 activation using 200 µM ATP with or without ginsenoside Rd was measured at 37 °C using YOPRO-1 (2 µM) as the membrane impermeant dye. Relative fluorescence units (RFU) were measured following excitation at 490 nm and emission recorded at 520 nm using a fluorescent plate reader (Flexstation 3). Black traces represent responses to agonist (ATP) and blue traces represent responses in presence of Rd + ATP. (B) Data was quantified as the slope of dye uptake curve. Error bars are S.E.M. *denotes P<0.05 using ANOVA with Dunnett's multiple comparison test.
Figure 4.8 CK enhances the sustained Ca\textsuperscript{2+} response associated with hP2X7 receptor activation.

(A) Intracellular Ca\textsuperscript{2+} responses were measured in Fura-2 AM loaded HEK-hP2X7 cells. Baseline values were recorded for 15 s and then ATP was applied and Ca\textsuperscript{2+} measured for 300 seconds. Ca\textsuperscript{2+} influx in the presence of ATP is shown in black circles and Ca\textsuperscript{2+} influx in the presence of CK + ATP is shown in red circles. (B) HEK-hP2X7 were treated with 200 μM ATP, 200 μM ATP + 0.01 % DMSO or 200 μM ATP + 10 μM CK and Fura-2 fluorescence measured over time. Quantitative measures sustained Ca\textsuperscript{2+} response (mean fluorescence between 100 and 300 seconds (baseline fluorescence between 0 – 15 seconds) in HEK-hP2X7 was plotted as bar graph. * denotes $P<0.05$, significant effect of CK; one-way ANOVA with Dunnett’s post-test.
Figure 4.9 There is no difference in the potentiating effect of selected ginsenosides on P2X7 mediated YOPRO-1 uptake in humans and rats.

(A) Concentration-response YOPRO-1 uptake curve of CK, Rd, Rb1 and Rh2 in ATP (200 µM) activated HEK-293 cells transfected with human P2X7. PPD (green) is included as a control. (B) Concentration-response curve of CK, Rd, Rb1, Rh2 in ATP (200 µM) activated HEK-293 cells transfected with rat P2X7 cells. PPD (green) is included as a control. Non-linear dose response curves of transformed data from 3 independent experiments were fitted using GraphPad Prism. Error bars represent S.E.M.
Figure 4.10 CK enhanced the ability of ATP to cause cell death in HEK-P2X7 cells.

Cell viability was measured using Cell Titre Aqueous ONE solution (Promega) after incubation HEK-hP2X7 cells with specific treatments for 24 hours. 3 mM ATP reduces cell viability 30% of the untreated controls. The effect is P2X7 mediated as the antagonist AZ10660120 (10 µM) reverts the cell viability to 81%. 500 µM ATP is considered as a non-lethal dose of ATP, which does not affect cell viability after 24 hours incubation period. In the presence of 10 µM CK, 500 µM ATP causes cells death equivalent to 3 mM ATP (app. 32%). The same effect is not observed at lower concentrations. Error bars represent S.E.M. * P<0.05 by one-way ANOVA (Tukey’s multiple comparison test).
4.4.6 Investigating effect of ginsenosides on P2X4

To gain a better insight into the specificity of ginsenosides towards P2X7, these bioactive saponins were tested on structurally homologous P2X4 and P2X2 receptors share structural homology with P2X7 and are known to form secondary permeability pathways similar to P2X7 (even though this property is most consistent and conspicuous for P2X7 (434). Unlike P2X7, P2X4 require a much lower concentration of ATP to be activated, with an EC\textsubscript{50} of 2.5 - 3 \textmu M (435, 436). To investigate ginsenosides on P2X4, a stable HEK-hP2X4 cell line was generated and the ginseng extract G115 (100 \mu g/ml) was tested using YOPRO-1 dye uptake as an indicator of receptor activity (as detailed in Chapter 3). Cells were either treated with 10 \mu M ATP alone or with G115 co-injected with ATP. The P2X4 antagonist 5-BDBD and the potentiator ivermectin (IVM) were used in order to ensure that P2X4-mediated responses were being measured. An increase in dye uptake was observed with cells treated with ATP + G115 and ATP + IVM compared to ATP alone (Figure 4.11). The ATP-induced YOPRO-1 uptake response was inhibited with 5-BDBD (Figure 4.11)

4.4.7 Effect of ginsenosides on P2X4 mediated dye uptake

Following this finding that G115 could enhance hP2X4 responses the 14 purified ginsenosides were tested. Similar to the observations made with P2X7, out of fourteen ginsenosides tested, four protopanaxadiol ginsenosides enhanced the dye uptake in the rank order Rd > CK > Rb1 > Rh2 (Figure 4.12).

4.4.8 Ginseng metabolite CK increases Ca\textsuperscript{2+} influx via P2X4 activation.

CK, the ginsenoside metabolite and its parent compound Rd, both of which produced maximum potentiation in case of P2X7, were studied in further detail for their effect on P2X4. A concentration response curve was obtained for dye uptake via P2X4 activated at different ATP concentrations (0.1-100 \mu M) in the presence of 10 \mu M CK or 5 \mu M IVM. 10 \mu M CK was seen
to cause a slight leftward shift in the ATP concentration-response curve, to increase the maximal response and to decrease the EC$_{50}$ value for ATP from 2.06 µM to 0.91 µM. IVM, the positive control also reduced the EC$_{50}$ for ATP to 1.6 µM (Figure 4.13).

4.4.9 Effect of selected ginsenosides on ATP concentration response curve in HEK-P2X4 cells
To better understand the potency of selected ginsenosides at P2X4, concentration responses were generated for the four protopanaxadiol ginsenosides. From the data in Figure 4.14, the EC$_{50}$ of the ginsenosides tested were 7.55 µM for Rd, 8.55 µM for CK, 10.10 µM for Rb1 and 5.8 µM for Rh2. Rh2 has the lowest effect on the ATP-induced maximal response but a potency comparable to other ginsenosides tested (Figure 4.14).

4.4.10 Effect of selected ginsenosides on Ca$^{2+}$ responses in HEK-P2X4 cells
The Ca$^{2+}$ permeability of hP2X4 is the highest among the P2X family (79) therefore it was pertinent to evaluate the effect of ginsenosides on intracellular Ca$^{2+}$ concentrations. ATP-induced activation of P2X4 leads to Ca$^{2+}$ influx which was tested experimentally using the ratiometric dye Fura 2-AM as previously described. The P2X4 receptor was activated using 1 µM ATP (concentration determined from the ATP dose response curve) and the ginsenosides were co-injected with ATP at a concentration of 10 µM each. The responses were measured on Flexstation 3 and the data was quantified as mean of sustained Ca$^{2+}$ response (100-300 seconds) observed after the initial transient peak response using baseline corrected data. Consistent with the dye uptake responses, the intracellular Ca$^{2+}$ was increased by ginsenosides in HEK-hP2X4 cells in the rank order of Rd > CK > Rb1 > Rh2 (Figure 4.15). Further experiments showed that 10 µM CK shifted the ATP concentration response curve leftwards and reduced the EC$_{50}$ value from 1.08 µM (95 % confidence interval 0.6 to 1.8 µM) to 0.3 µM (95 % confidence interval 0.1 to 0.5 µM) (Figure 4.16).
4.4.11 Effect of ginsenosides on P2X4 inward currents using high throughput IonFlux-16 automated patch clamp system.

To investigate the effect of ginsenosides on P2X4 channel, the automated patch clamp FluxIon system was used. The FluxIon plates were primed before adding the HEK- P2X4 cells to them. The cells were prepared at a cell density of 4-10 x 10⁶ cells/2 ml extracellular solution, so that the final cell density is 2.5 x 10⁶ cells/ml. Cell suspension (250 µl) was added to each “IN” well of the Ion-flux 16 plate (Figure 2.2). The plate was loaded on to the FluxIon automated patch clamp system. The drug solutions were prepared in 2 x concentrations. ATP was applied first and the responses (current amplitude in pA) were measured. Then the cells were washed out with running buffer for 2 minutes. ATP was then reapplied in the presence of various ginsenosides. Current amplitudes were then measured as an average current of 20 cells for each condition using FluxIon software. Data was expressed as fold change in current amplitude.

P2X4 antagonist 5-BDBD was able to inhibit the current amplitude by 74.3 %. In the presence of 10 µM CK and 10 µM Rd, ATP mediated increase current amplitude was increased by ~ 3 folds ( > 50 % increase in comparison to 0.01 % DMSO control) in HEK-P2X4 cells. Rh2 caused a 44.7 % increase in current amplitude but Rb1 was not seen to cause an increase in channel activity in these settings (Figure 4.17).

4.4.12 Effect of P2X4 and ginsenosides on cell viability

To test whether P2X4 potentiation by ginsenosides leads to initiation of apoptotic cell death, a cell viability assay similar to the one described above for hP2X7 was performed. In these experiments cell viability was measured as end-point absorbance of MTS solution (490 nM) for each treatment and data was quantified as percentage of untreated control (designated as 100 % viability). Unlike the observations made with HEK-hP2X7 cells though, no significant difference was seen in the viability of HEK-P2X4 cells at any ATP concentration tested (Figure 4.18). A high 3 mM concentration of ATP is considered to be a lethal concentration of ATP.
for P2X7 containing cells. In HEK-293 cells expressing only hP2X4, 3 mM ATP did not cause any significant reduction in cell viability (cell death) (Figure 4.18). The presence of 10 μM CK together with ATP did not reduce cell viability (Figure 4.18).
Figure 4.11 Effect of ginseng extract G115 on ATP-induced dye uptake in HEK-293 cells expressing human P2X4

(A) YOPRO-1 uptake was induced in HEK-hP2X4 expressing cells by the addition of 5 µM ATP (denoted by the arrow) in low divalent physiological solution. Control YOPRO-1 uptake to ATP is shown in black circles. The response was inhibited by P2X4 antagonist 5-BDBD (20 µM) and potentiated by 5 µM IVM, a positive allosteric modulator of P2X4, shown here in brown circles and black open circles respectively. Fluorescence was measured for 50-300 seconds on Flexstation 3 (Molecular Devices). (B) Bar charts displaying data from 3 separate experiments. The data was quantified using area under the dye uptake curve, baseline corrected. The P2X4 mediated dye uptake was significantly attenuated by 5-BDBD and facilitated by IVM. 100 µg/ml G115 increased dye uptake via ATP activated P2X4. Error bars represent S.E.M ** denotes P < 0.05 by one-way ANOVA (Dunnett’s post-test).
Figure 4.12 Effect of ginsenosides on ATP-induced dye uptake in HEK-293 cells expressing human P2X4

YOPRO-1 uptake in HEK-hP2X4 cells in presence of 5 µM ATP (denoted by the arrow), ATP + PPD1 and ATP + CK in low divalent buffer solution. The ginsenosides were co-injected with agonist ATP. Fluorescence was measured for 50-180 seconds on Flexstation 3. Ginsenosides Rd, Rb1, Rh2 and CK (10 µM ATP) were seen to potentiate the P2X4 mediated dye uptake Data has been quantified as area under dye uptake curve (50-180 sec). Bar graphs show data from 3-5 independent experiments. Error bars represent S.E.M, * denotes $P<0.05$ by one-way ANOVA (Dunnett’s post-test).
Figure 4.13 ATP-induced dye uptake in HEK-293 cells expressing human P2X4 in the presence of varying doses of CK

CK increases the potency and efficacy of ATP binding to P2X4. The EC₅₀ of ATP is lowered from 1.92 µM to 1.215 µM in the presence of 10 µM CK. Various concentrations of ATP (1 µM - 100 µM) were used to activate the P2X4 receptor and the activation was measured by YOPRO-1 uptake on Flex station 3. Controls are shown here in black dots, the dye uptake in presence of 10 µM CK is shown in red and in presence of P2X4 potentiator ivermectin (IVM) is shown in blue. The data is taken from 3 independent experiments and transformed data has been fitted using non-linear fit of regression. The error bars represent S.E.M.
Figure 4.14 Concentration dependent effect of ginsenosides on ATP-induced dye uptake in HEK- human P2X4 cells

Concentration dependent responses of selected ginsenosides on ATP responses in HEK-P2X4 cells- Different concentrations (0.1-50 µM) of ginsenosides were tested for their effect on YOPRO-1 uptake in HEK-P2X4 cells stimulated at fixed concentration (5 µM) of ATP. The EC\textsubscript{50} of different ginsenosides was in the rank order Rb1 > Rd > Rh2 > CK with values being 0.11 µM, 7.54 µM, 5.8 µM and 8.5 µM. The data is taken from 3 independent experiments and transformed data has been fitted using non-linear fit of regression. The error bars represent S.E.M.
Figure 4.15 CK enhances the sustained Ca$^{2+}$ response associated with hP2X4 receptor activation.

(A) Intracellular Ca$^{2+}$ responses were measured in Fura-2AM loaded HEK-hP2X4 cells. Baseline values were recorded for 15 s and then ATP was applied. Ca$^{2+}$ influx in presence of ATP is shown in black and Ca$^{2+}$ influx in presence of ATP + CK shown in red (B) HEK-hP2X4 were treated with 1 μM ATP or 1 μM ATP + 10 μM ginsenosides and Fura-2AM responses measured over time. Intracellular Ca$^{2+}$ in ATP activated HEK-hP2X4 is shown in black bar. 10 μM CK and Rd significantly increase P2X4 mediated the Ca$^{2+}$ influx. Error bars denote S.E.M; * denotes $P<0.05$, significant effect of CK; one-way ANOVA with Dunnett's post-test.
Figure 4.16 CK acts a positive modulator of P2X4 receptor

CK acts a positive modulator of P2X4 receptor by shifting the ATP dose response towards left. The data is quantified as sustained Ca$^{2+}$ response (mean fluorescence between 100 and 300 s). The data was taken from 10-12 wells from 2 independent experiments and transformed data was fitted using non-linear fit of regression. The EC$_{50}$ of ATP for P2X4 was reduced from 1.08 µM to 0.27 µM in the presence of 10 µM CK. The error bars represent S.E.M.
Figure 4.17 Effect of ginsenosides on current amplitude in HEK-hP2X4 cells

The HEK- P2X4 cells were used at a cell density of 4-10 x 10^6 cells/2 ml of extracellular solution and added to the primed IonFLux-16 plates. The final cell density is 2.5 x 10^6 cells/ml. Cell suspension (250 µl) was added to each “IN” well of the IonFlux 16 plate. The plate was loaded on to the IonFlux automated patch clamp system. The drug solutions were prepared in 2 x concentrations. 5-BDBD was prepared in running buffer. ATP was applied first and the responses (current amplitude) were measured. Then the cells were washed out with running buffer for 2 minutes. ATP was then reapplied in the presence of various ginsenosides. Current amplitudes were then measured for each condition using IonFlux software. Data from 3 independent experiments was collated and was expressed as fold change in current amplitude. Error bars represent S.E.M; *denotes P<0.05, One-way ANOVA, Dunnett’s multiple comparison test.
Figure 4.18 ATP does not induce cell death in HEK-hP2X4 cells

Cell viability was measured using Cell Titre Aqueous ONE solution (Promega) after incubation HEK-hP2X4 cells with specific treatments for 24 hours. 3 mM ATP had no effect on cell viability. A range of different concentrations (5µM-3mM) was tested on HEK-P2X4 cells in the presence of CK and P2X4 antagonist 5-BDBD (20 µM). No changes were observed in cell viability under any conditions after 24 hours incubation period. Error bars represent S.E.M ns denotes not significant, one-way ANOVA (Tukey’s multiple comparison test).
4.4.13 Role of ginsenosides in modulation of P2X2 receptor function

Pharmacological investigations were carried out on another member of the purinergic P2X family, the hP2X2 receptor. The full sized P2X2 receptor is called P2X2a to differentiate it from its multiple splice variants which are found naturally in humans (for instance, the splice variant P2X2b has missing 69 residues in its C-terminal domain) (437). For measuring YOPRO-1 uptake, the P2X2a receptor was activated by 5 µM ATP (Figure 4.19 A, B) in a low divalent buffer. Ginsenosides CK, Rd and Rh1 were seen to increase the dye uptake (Figure 4.15). Rb1 on the other hand did not alter the P2X2a response. For Ca\(^{2+}\) influx measurement, 1µM ATP was used to activate the receptor. Similar to the results obtained from dye uptake experiments, CK caused a robust increase in Ca\(^{2+}\) influx through activated P2X2a but other ginsenosides Rd, Rb1 and Rh2 did not mimic this potentiating affect (Figure 4.20 A, B). This observation was different from expected as dye uptake and intracellular Ca\(^{2+}\) measurements are generally considered as the indicators of P2X2 receptor activity. Therefore, an increase in dye uptake with Rd and Rh2 would generally translate into increase in Ca\(^{2+}\) influx as well, which was not seen in these experiments. Since CK is the drug of interest in this study and had shown an effect on P2X2 mediated dye uptake as well as Ca\(^{2+}\) responses in these experiments, 10 µM CK was tested on ATP dose response using both YOPRO-1 and Fura-2 measurements (Figure 4.15 C, D). CK was seen to reduce the EC\(_{50}\) of ATP in both measures. For YOPRO-1 uptake, EC\(_{50}\) of ATP was changed from 0.6 µM to 0.25 µM by CK, with a definite leftward shift (Figure 4.16 A). For intracellular Ca\(^{2+}\), the EC\(_{50}\) for ATP alone in P2X2a expressing cells is lowered from 3.03 µM to 2.67 µM in the presence of CK (Figure 4.16 B).
Figure 4.19 Effect of ginsenosides on P2X2a dye uptakes in transiently transfected HEK-293 cells

(A) YOPRO-1 uptake was measured in HEK-293 cells transiently transfected with P2X2a plasmid. Non-transfected cells were used a negative control (mock shown in grey patterned bar). Since, no specific antagonist of P2X2a is available; P2X7 blocker AZ10660120 (grey bar) and P2X4 potentiator IVM were used to rule out the possibility of a P2X7 or P2X4 response. Data was quantified as area under the dye uptake curve, baseline corrected. (B) Dye uptake by ATP activated HEK-P2X2a cells were tested by co-injecting ginsenosides with ATP. CK and Rd at 10 µM concentration potentiated the dye uptake significantly. The bar chart display data from 3 separate experiments. Rb1 and Rh2 did not cause a significant change in dye uptake. The data was quantified using area under the dye uptake curve, baseline corrected. Error bars represent S.E.M. * denotes $P<0.05$ by one-way ANOVA (Dunnett’s post-test).
Figure 4.20 Effect of ginsenosides on P2X2a Ca\(^{2+}\) influx in transiently transfected HEK-293 cells

(A) Ca\(^{2+}\) influx was measured in HEK-293 cells transiently transfected with P2X2a plasmid using Ca\(^{2+}\) sensitive ratiometric dye Fura-2AM. Data was quantified as mean of sustained Ca\(^{2+}\) response from 100 – 300 seconds. The mean of minimum response (0-15 seconds) was deducted from the sustained response. (B) Intracellular Ca\(^{2+}\) concentrations in ATP activated HEK-P2X2a cells were tested by co-injecting ginsenosides with ATP. 10 µM CK potentiated the dye uptake significantly. The bar chart displaying data from 3 separate experiments. Rd, Rb1 and Rh2 did not cause a significant change in intracellular Ca\(^{2+}\) levels. Data was quantified as mean of sustained Ca\(^{2+}\) response from 100 – 300 seconds. The mean of minimum response (0-15 seconds) was deducted from the sustained response. Error bars represent S.E.M * denotes \(P<0.05\) by one-way ANOVA (Dunnett’s post-test)
Figure 4.21 Effect of ginsenosides on P2X2a Ca\(^{2+}\) influx in transiently transfected HEK-293 cells

(A) Concentration dependent response of ginsenosides on YOPRO-1 uptake was measured in HEK-P2X2a cells. The EC\(_{50}\) of ATP when measured by dye uptake is lowered from in the presence of 10 µM CK. Various concentrations of ATP (0.01 µM - 50 µM) were used to activate the P2X2a receptor and the activation was measured by YOPRO-1 uptake on Flexstation 3. Data was quantified as area under dye uptake (50-300 seconds), baseline corrected. The data is taken from 3 independent experiments and transformed data has been fitted using non-linear fit of regression. The error bars represent S.E.M; P<0.05. (B) Various concentrations of ATP (0.01 µM - 50 µM) were used to activate the P2X2a receptor and the activation was measured by Ca\(^{2+}\) using ratiometric dye Fura-2AM on Flexstation 3. The EC\(_{50}\) of ATP is lowered in the presence of 10 µM CK. The data is taken from 3 independent experiments and transformed and fitted using non-linear regression.
4.4.14 Effect of ginsenosides in modulation of P2Y1 and P2Y2-mediated Ca\(^{2+}\) responses

Finally, the ginsenosides were tested on the metabotropic P2Y receptor which is expressed in immune cells along with the other P2X receptors tested in this study. P2Y receptors are G coupled proteins (GPCRs) for extracellular nucleotides. The receptors were activated using either 10 µM ATP (which is an agonist for both P2Y1 and P2Y2), 10 µM ADP (a P2Y1 agonist) or 10 µM UTP (a P2Y2 receptor agonist). The peak was measured from 0-100 seconds in the presence and absence of CK. The ginsenosides were found to have no significant effect on P2Y1 and P2Y2 mediated Ca\(^{2+}\) release from intracellular stores (Figure 4.21).

Taken together, these results suggest that the selected ginsenosides CK, Rd, Rb1 and Rh2 potentiate P2X7 mediated responses but are not completely specific for this receptor as potentiating effects of these ginseng saponins are observed in the close relatives P2X4 and P2X2a. However, there is considerable difference in the magnitude of potentiation of these responses between the different receptors. In all these experiments, the major in vivo metabolite CK showed a consistent behaviour of potentiating the P2X receptor responses. To compare the magnitude of the CK effect, the data from the dye uptake and Ca\(^{2+}\) influx experiments for P2X7, P2X4 and P2X2a was plotted on to one graph. The response for ATP treatment was designated as 100% and the ATP + CK response for each of these receptors was compared to their own control. Fold change was calculated for each of these receptors using the following formula

\[
\text{Fold change} = \frac{\text{Area under curve }_{\text{Treated}} - \text{Area under curve }_{\text{control}}}{\text{Area under curve }_{\text{control}}}
\]

It was observed that the magnitude of increase in receptor potentiation by ginsenosides CK was in the rank order P2X7 >> P2X2a > P2X4. Thus, these pharmacological studies indicate even though the ginsenosides are not specific for P2X7 responses alone, they definitely elicit their largest effect via this receptor (Figure 4.22).
Figure 4.21 Selected ginsenosides do not alter P2Y mediated Ca\(^{2+}\) influx

HEK-293 cells express P2Y1 and P2Y2 endogenously. Effect of ginsenosides on P2Y mediated Ca\(^{2+}\) influx was measured using Fura-2 AM. The receptors were activated using either 10 µM ATP (agonist for both P2Y1 and P2Y2), 10 µM ADP (P2Y1 agonist) or 10 µM UTP (P2Y2 receptor). Cells were treated with agonist, agonist + 10 µM ginsenosides or agonist + antagonist (suramin). (A) Raw traces of intracellular Ca\(^{2+}\) measurement following activation by agonist. Baseline measurements were done for 15 seconds, after which agonist was injected. Black open squares are responses in presence of non-specific P2Y inhibitor suramin and green triangles represent Ca\(^{2+}\) responses in presence of agonist and aglycone PPD. (B) Data was quantified as mean of peak response (15–200 sec). (B) represents the P2Y mediated Ca\(^{2+}\) influx in the presence of ATP. (C) represents the P2Y mediated Ca\(^{2+}\) influx in the presence of ADP. (D) represents the P2Y mediated Ca\(^{2+}\) influx in the presence of UTP. Agonist (ATP, UTP or ADP) responses are represented as black bar in graphs B,C and D. Ginsenosides CK, Rd, Rb1 and Rh2 have no significant effect on P2Y mediated intracellular Ca\(^{2+}\) influx. Data analysis done by one-way ANOVA with Dunnett’s post-test.
Figure 4.22 Comparative analyses of ginsenosides on P2X7, P2X4 and P2X2a mediated YOPRO-1 uptake and Ca$^{2+}$ responses

(A) HEK-P2X7 cells activated using 200 µM ATP (EC50) with and without 10 µM CK. The amount of YOPRO-1 uptake was quantified as area under the dye uptake curve (40-300 sec). The bar graph represents the fold change between controls and with CK after normalising controls as 100% response. Fold change was calculated using the formula (Area under curve$_{Treated}$/ Area under curve$_{control}$/ Area under curve$_{control}$). 10 µM CK potentiate dye uptake in ATP activated HEK-P2X7 cells by 4.9 folds. Similarly, the HEK-P2X4 cells were stimulated at 5 µM (EC$_{50}$) in the absence of ATP and also with CK + ATP. The responses are increased by 0.5 folds. For HEK-P2X2a (EC$_{50}$ - 5 µM) this increase was 1.3 folds. Thus, CK potentiates P2X7 mediated dye uptake most. (B) The HEK-P2X7 cells were activated using 200 µM ATP (EC$_{50}$) with and without 10 µM CK. The amount of Ca$^{2+}$ was quantified as Fura 2-AM fluorescence (100-300 sec). The bar graph represents the fold change between controls (ATP) and with CK after normalising controls as 100% response. 10 µM CK potentiate dye uptake in ATP activated HEK-P2X7 cells by 4.6 folds. Similarly, the HEK-P2X4 cells were stimulated at 5 µM in the absence of ATP and also with CK + ATP. The responses are increased by 0.7 folds. For HEK-P2X2a (EC$_{50}$ -5 µM) this increase was 1.3 folds. Thus, CK potentiates P2X7 mediated Ca$^{2+}$ influx maximally in comparison to other purinergic receptors tested.
4.6 Key Points

➢ Out of 14 ginsenosides tested, 4 ginsenosides potentiate P2X7 mediated dye uptake and Ca\(^{2+}\) influx in rank order CK > Rd > Rb1 > Rh2.
➢ Presence of at least one carbohydrate group seems to be essential for potentiation as aglycone PPD has no effect on P2X7 responses.
➢ CK increase the capability of P2X7 to cause cell death.
➢ Selected ginsenosides tested are not specific for P2X7 as closely related P2X4 and P2X2a are also potentiated.
➢ In comparison to P2X4 and P2X2, the ginsenosides are more efficacious towards P2X7 responses.
➢ Metabotropic P2Y receptors are not affected by ginsenosides Rd, Rb1, Rh2 and ginseng metabolite CK.

4.7 Discussion

In this study, a significant increase in P2X7 receptor responses was observed in the presence of certain ginseng saponins (Rb1, Rh2, Rd and CK). To observe an increase in P2X responses by these selected ginsenosides activation of P2X7 receptors by orthosteric agonists (ATP, BzATP) was found to be necessary. These effects were characterized in a HEK-293 cell line expressing P2X7 receptors. As a result of the P2X7-ginsenoside interaction an increase Ca\(^{2+}\) influx and a subsequent decrease in cell viability to lower concentrations of ATP was observed. Taking into consideration the widespread distribution of P2X7 channels on immune cells and the fact that effects could be observed in the submicromolar range with one of the principal metabolites of ginseng CK (from the electrophysiology data obtained in the lab and published in 2015) (438), it is possible that this mechanism may account for some of the reported immune modulatory actions of ginseng in vivo. In case of P2X7 receptor responses it appears that this potentiation is exclusively exhibited by glycosylated PPD ginsenosides and can be observed in
the low to sub-micromolar range (EC\textsubscript{50} of 0.5–1.1 \(\mu\)M), the probability of this effect being a result of a non-specific interaction due changes in membrane fluidity (as has been suggested for certain ginsenoside actions reflecting the amphipathic nature of these steroid-like saponins), is ruled out.

The increase in P2X7 receptor activity by the PPD class of ginsenosides is reported for the first time in the present study, however there have been previous reports of some PPD ginsenosides such as Rc and Rd potentiating GABA\textsubscript{A} and glycine currents in Xenopus oocytes (439, 440). In the studies mentioned above, the concentrations of ginsenosides required to elicit measurable enhancement of receptor activity were much higher (approximately 50 \(\mu\)M), but the results indicated that the PPD class was more potent than PPT ginsenosides. Ginsenoside Rg3 was also found to enhance the activity of GABA channels containing the \(\gamma2\) subunit. Nah (2014) has extensively reviewed the effect of various ginsenosides on membrane receptors and ion channels (404). There are a number of studies indicating the inhibitory effect of ginsenosides on voltage gated Ca\textsuperscript{2+}, K\textsuperscript{+}, Na\textsuperscript{+} and hERG K\textsuperscript{+} and KCNQ K\textsuperscript{+} channels (441-444). The present study indicates that the aglycone compounds PPD and PPT were not effective at increasing P2X7 activity which indicates that to have an effect on the P2X7 receptor function, the presence of at least one sugar moiety may be essential. Interestingly, both PPD and PPT aglycones have been shown to inhibit rather than potentiate GABA\textsubscript{A} currents (445). However, no inhibitory effects on P2X7 receptor responses with any ginsenoside compound were observed in this study. Further investigations are required to determine if there are any shared structural features between the ginsenoside binding sites on P2X7 and GABA\textsubscript{A} channels. This may also apply to HERG (K\textsubscript{v}11.1) channels, as PPD ginsenosides were typically more effective than PPT ginsenosides in potentiating tail currents and PPT/PPD aglycones were ineffective (443).

Glycosylated PPD ginsenosides, such as the principal ginseng metabolite CK, are novel potent positive allosteric modulators of human P2X7 receptors. Several other positive modulators of
P2X7 receptors have been described including clemastine, tenidap, polymixin B and Ivermectin (210, 446-449). The mechanism of CK on P2X7 channels shares some similarities with clemastine, in that the action is rapid, reversible, Ca\(^{2+}\) and voltage-independent and likely use an extracellular site. Similar to CK, modulators such as clemastine had no direct effects on P2X7 channels and required the presence of the agonist ATP (447). Although in the above mentioned study, it was reported an augmented alteration in reversal potential occurring over tens of seconds, reflecting an increased rate in the permeability to NMDG\(^+\) in the presence of ATP, they recognized that this cannot account for the rapid (<1 s) potentiating effect and reversibility of clemastine (447). The most plausible explanation, which may also be applicable to PPD ginsenosides, is that such modulators increase the mean-open time of P2X7 channels. Furthermore, such a net increase in channel activation is known to accelerate pore dilation.

An important consequence of the PPD ginsenoside action on P2X7 channels is enhanced sustained Ca\(^{2+}\) influx in HEK-hP2X7 cells. The use of CK as a positive allosteric modulator of P2X7 receptors reduces the concentration of ATP required to generate a sustained Ca\(^{2+}\) response. Many downstream consequences of P2X7 receptor activation depend on sustained Ca\(^{2+}\) signalling (136). With regard to cell viability, brief additions of high concentrations of ATP (<5 min, < 1 mM) can lead to a transient ‘pseudoapoptosis’ that does not lead to cell death (450) or to a delayed cell death occurring after a number of hours (107). Higher concentrations of ATP and/or prolonged applications on the other hand lead to cell death within minutes because of massive Ca\(^{2+}\) influx. In contrast, lower concentrations of ATP (<1 mM) can have the opposite effect, stimulating proliferation and prolonging cell survival (202). Consistent with this, the present study has shown that enhancing Ca\(^{2+}\) influx via P2X7 receptors in HEK-hP2X7 cells through the use of CK can effectively convert a sublethal dose of 500 μM ATP into a lethal concentration as measured by a significant decrease in cell viability after 24 h. However, given the fact that the timing and extent of Ca\(^{2+}\) influx via P2X7 receptors can lead to different functional outcomes, further studies are warranted with different
ATP/ ginsenoside combinations and examination of other parameters in addition to cell viability. Keeping this mind, the effect of ginsenosides on cell viability and signalling pathway involved has been evaluated using macrophages (J774 cell line and rat macrophages). The results of this study have been discussed in Chapter 5.

After establishing the potentiating effect of ginseng metabolite CK on P2X7, the selectivity of CK and other selected ginsenosides (Rd, Rb1 and Rh2) was tested on structurally homologous P2X receptors; P2X4 and P2X2a. P2X4 receptors are expressed in immune cells along with P2X7. Functional coordination between P2X7 and P2X4 is well characterised (451, 452). Here it was observed that all selected ginsenosides (more so for CK and Rd that Rb1 and Rh2) increased responses via P2X4 as well as P2X2 activation, showing that ginsenoside action was not exclusive for P2X7 but targeted closely related purinergic family members (Figure 4.8-4.12). However, the increase was approximately 0.5 fold for P2X4 and 1.3 fold for P2X2 in comparison to P2X7 responses which increase approximately 5 fold in the presence of ginsenosides.

Since there is evidence that P2X4 can interact with and regulate P2X7 dependent downstream signals (453), the effect of CK on cell viability in HEK-P2X4 cells was tested in a manner similar to the one used for HEK-P2X7 cells. No change in viability was observed in the presence of CK + ATP in HEK-293 cells expressing P2X4 receptor (Figure 4.11), suggesting the lack of involvement of P2X4 in cell death pathway in these cells.

A number of positive allosteric modulators of P2X4 such as Zn^{2+}, cibacron blue, ivermectin, doramectin, moxidectin and a few others have been identified and characterised (454). The data from the present study suggests that the positively modulation of P2X4 responses by selected ginsenosides may be similar to Ivermectin (IVM), a well characterised positive allosteric modulator of P2X4. Among the purinergic receptors tested, P2X4 is most sensitive towards IVM (314), although there is some evidence that IVM also increases human P2X7
responses to a certain extent. IVM (448), the macrocycline lactone derivative of naturally occurring avermectin B1, is known to act from the extracellular side in order to potentiate P2X4 currents and delay channel desensitization. It is suggested that IVM binds to a high affinity site on the transmembrane domains. Ivermectin has been found to modulate the sensitivity of P2X7/P2X4/Panx-1 pathway towards extracellular ATP leading to induction of autophagic and inflammatory cell death in breast cancer cells (455). IVM has been used as a positive control in the experiments conducted in the present study and it has been observed that the magnitude of P2X4 potentiation caused via G115 is similar to IVM (Figure 4.7).

Alcohol acts a negative allosteric modulator of P2X receptors (456) and a decrease in P2X4 gene expression has been observed in brain on alcohol consumption (182, 457). Studies in rodents and humans have shown that IVM binds to P2X4 on a different site than ethanol and reduces the alcohol induced debilitating effects by elevating the P2X4 responses to normal levels (458). Furthermore, there are reports showing that ginseng extract G115 reduces ethanol induced depression in mice (459). A randomised cross over study has shown that red ginseng can alleviate alcohol consumption and hangover related symptoms in men (460). There is also a mounting body of evidence that purinergic receptors contribute to neuronal survival as well as function (461). It has been found that P2X4 expression decreases in neuronal cells in Alzheimer’s disease (462). There are a number of studies supporting the protective role of ginseng and its active constituents in this debilitating neurodegenerative disorder (463-465). Taken together, these studies suggest that potentiation of P2X4 responses by selected ginsenosides may be involved in the beneficial effects of ginseng in relieving alcohol related disorders as well as Alzheimer’s disease.

Among the 7 P2X subtypes, P2X4 is the most highly expressed in vascular endothelium (466, 467). ATP release from vascular endothelial cells results in modulation of P2X4 responses leading to increased Ca2+ influx (468). A loss-of-function mutation in P2X4 has been
associated with decrease in P2X4 responses leading to increased pulse pressure, an independent risk factor of cardiovascular diseases. People inheriting the defective P2X4 gene may be more vulnerable to cardiovascular disorders (469). Potentiation of P2X4 responses by ginsenosides in people inheriting mutated or dysfunctional P2X4s may be a useful way of preventing cardiovascular disease conditions such as atherosclerosis and thrombosis.

The present study provides evidence in support of the positive allosteric effect of protopanaxadiol ginsenosides on hP2X2 receptor. P2X2a is known to be distributed in central and peripheral nervous system (470). Neurotransmitters such as nicotine, 5-hydroxytryptamine (5-HT), noradrenaline, adenosine, bradykinin and histamine are known to increase P2X2a responses at a high concentration of 100 µM (471). Ginsenosides in the present study potentiate these responses at a much lower concentration (10 µM being the highest concentration tested). Increase in P2X2a responses can lead to increase in sensory nerve sensitivity. P2X2a receptors are also extensively distributed in cochlear cells in the ears and play a role in sound transduction. Under stress conditions, the amount of ATP increases, causing P2X2a activation. This ATP-P2X2a-mediated shunt conductance can contribute to change in hearing sensitivity and temporary hearing loss phenomena and also serve a protective role by decoupling the cochlear amplifier in response to cochlear stressors (472). The reported protective effect of ginseng towards hearing loss could be explained by increase in sensitivity of cochlear cells towards stressors via P2X2a activation leading to decoupling the positive feedback mechanism within the cochlea that provides acute sensitivity in the mammalian auditory system.

Selected ginsenosides when tested were found to have no effect on the activity of these receptors. These results indicate that the ginsenosides tested may be targeting only purinergic ion channels and not their G-protein coupled receptor counterparts.

The results presented in this chapter may also shed some light on a possible structure function relationship between these ginsenosides and their potency towards the receptors. CK and Rd
have a glucose molecule attached on their C-20 (R2 position) carbon atom while Rh2 and Rb1 have -H at the C-20 position. So, even though Rh2 and Rb1 potentiate P2X responses significantly but this increase is not as effective as CK and Rd. Thus, from these results it may be suggested that the glucose group present on C-20 may facilitate the binding of ginsenosides on P2X receptors and increasing the potency of agonist ATP for its receptor. Overall, the data from the present study provides evidence that selected ginsenosides can target purinergic receptors to positively modulate the receptor responses.

4.8 Conclusions
From the present study, it can be suggested that ginseng metabolite CK is a potent allosteric modulator of purinergic ion channels expressed in immune cells, being most efficacious towards P2X7. Presence of glucose in the molecular structure may to be essential for ginsenosides to elicit an increase in P2X7 responses, as aglycone PPD has no effect on the magnitude of the responses elicited by this receptor. It could also be suggested that the efficacy of a particular ginsenoside in potentiating P2X response is due to the presence of a glucose moiety on C-20 carbon of its steroid ring, as CK and Rd are more effective in enhancing responses than Rb1 and Rd. The identification and characterisation of novel allosteric modulators of the purinergic system not only provide a credible explanation to the certain immunomodulatory properties of ginseng but may also hold a promise for development of new therapeutic tools in order to target purinergic receptors expressed in immune cells involved in various pathophysiological conditions.
CHAPTER 5

SELECTED GINSENOSIDES MODULATE P2X7 RECEPTOR
RESPONSES IN MACROPHAGES

5.1 Introduction

Macrophages are special cells of the innate immune system, which are involved in acute as well as chronic inflammatory processes. In case of infection, macrophages migrate to the site of invasion and initiate their antimicrobial activity (473). After neutralizing the infection, the process of repair and resolution is also taken care of by these cells. Macrophages have the capability to turn from pro-inflammatory to anti-inflammatory depending upon the cytokine profile in their microenvironment (474). The inflammatory processes are tightly regulated, and any imbalance can result in development of chronic inflammatory state. Most current therapies target the initiation and progression phase of inflammatory process, but it has been strongly suggested that intervention at repair and resolution phase of inflammation may be a better approach in the conditions related to chronic inflammation (475).

P2X7 is endogenously expressed in macrophages and is the most extensively studied purinergic receptor from the immunological perspective (262). High concentrations (in millimolars) of ATP are released by infected, injured or dying cells in the extracellular milieu where it acts on the P2X7, thereby triggering an inflammatory response in the cells. The eATP-P2X7 interaction is widely accepted as one of the most important signalling triggers in infectious and inflammatory diseases (54).

The ATP- P2X7 liaison leads to K⁺ efflux, NLRP3 inflammasome assembly, caspase-1 and pro-IL-1 β secretion (476). The increase in Ca²⁺ influx following P2X7 activation is known to affect the mitochondrial membrane potential causing an increase in ROS production, which
may further activate the caspase 3/7 leading to an increase in cell death via apoptosis (477, 478). Modulation of P2X7 responses can have a significant effect on the associated signalling pathways.

In the previous chapter, the effect of selected ginsenosides on P2X receptors expressed in HEK-293 cells was investigated. This study provided us with an opportunity to look at P2X receptor responses to ginsenosides in the absence of any other interacting purinergic receptors. From the previous study, it was established that ginsenosides can modulate P2X responses. The next conceptual step was to investigate the effect of these ginsenosides in a system, which was physiologically more relevant, such as macrophages, where these receptors are co-expressed endogenously along with the signalling pathways they regulate. Therefore, in the present chapter the selected ginsenosides were tested for their effect on P2X mediated physiological responses using a J774 macrophage cell line as well as primary peritoneal macrophages.

Lipopolysaccharide (LPS) is a major component of Gram-negative bacteria (also called bacterial endotoxin) and it acts as primary inflammatory stimuli after binding to TLR-4 receptors expressed on macrophages (479). Humphrey and Dubyak first reported enhancement of P2X7 mediated cellular responses by LPS in 1996 (480). It is now well established that LPS-TLR4 binding leads to activation of the NLRP3 inflammasome cascade, causing maturation and release of the pro-inflammatory cytokine IL-1β and IL-18 (316). However, this LPS treatment stimulates only a slow rate of caspase-1 activation. Caspase-1 is known to catalyse the maturation and release of pro-inflammatory cytokines. A slow rate of caspase-1 activity by LPS leads to the accumulation of the pro-IL1β in the macrophages. It has been observed that for an accelerated caspase-1 activity, efflux of K+ ions are required from monocytes and macrophages. Recent studies have shown that P2X7 involvement is critical for the inflammasome activation to occur as P2X7 membrane pore leads to K+ ion efflux, which causes
the inflammasome assembly to occur. Therefore, modulation of P2X7 receptor can have an effect on LPS mediated pro-inflammatory effects. This aspect has been assessed in this study, where cells pre-exposed to LPS are then incubated with ginsenosides in order to evaluate changes in the resulting P2X7 mediated inflammatory pathways.

Ginseng has been used traditionally as an immune-boosting tonic with little understanding of target receptors. This study provides evidence that ginsenosides target P2X receptors and alter the signalling pathways mediated by them and thus provides an explanation to some of their immune-modulatory properties. The results from this study may be helpful in developing these nutraceuticals as therapeutic tools for targeting P2X mediated pathological conditions such as rheumatoid arthritis, bronchitis, chronic obstructive pulmonary disorder (COPD) and cancer.

5.2 Research Question

In this study, the effects of selected protopanaxadiol ginsenosides CK, Rd, Rb1 and Rh2 on responses mediate by ATP-gated P2X7 receptors in macrophages to characterise these newly identified pharmacological modulators was investigated.

5.3 Results

5.4.1 J774 macrophages express functional P2X7

In this chapter, the modulatory effect of ginsenosides was tested in a physiologically suitable environment by using a J774 macrophage cell line. J774 macrophage cell line is an immortal cell line derived from the ascites from peritoneal cavity of a mouse (https://www.atcc.org/Products/All/TIB-67.aspx). The suitability of this cell line as an ideal model system for studying macrophage responses has been studied and it has been found that the J774 macrophages cells respond in a manner comparable to primary macrophages and therefore are a convenient and a reliable mode for investigating signalling pathways distinctive to macrophages (481-483). Previous reports have confirmed the endogenous expression of
P2X7 in the J774 macrophages (212, 484-488). It was pertinent to establish the functionality of these receptors in J774 cell line used in this study. For this purpose, the ATP-P2X7 mediated dye uptake and Ca\(^{2+}\) influx was measured using YOPRO-1 and Fura 2-AM respectively. Following activation by ATP, there was a significant increase in dye uptake indicating the P2X7 mediated membrane pore formation. Attenuation of dye uptake was observed in the presence of P2X7 antagonist AZ10606120. No increase in YOPRO-1 fluorescence was seen in the absence of ATP (Figure 5.1 A and Figure 5.1 B). Furthermore, intracellular Ca\(^{2+}\) concentrations measured using ratiometric Ca\(^{2+}\) indicator Fura 2-AM showed an increased Fura-2 fluorescence on ATP application and inhibition of this fluorescence in presence of antagonist (Figure 5.1 C and Figure 5.1 D). These results validated the presence of a functional P2X7 in the J774 macrophage cell line employed in this study.
Figure 5.1 Expression of functional P2X7 receptor in J774 macrophages
(A) YOPRO-1 uptake was induced in J774 macrophages by the addition of 500 µM ATP at 40 seconds (denoted by the arrow) in low divalent physiological solution. An increase in YOPRO-1 fluorescence was observed on addition of ATP (black circles). P2X7 antagonist AZ10606120 inhibited the response (grey triangles). Fluorescence was measured for 50-300 seconds on Flexstation 3 (Molecular Devices). (B) The data was quantified using area under the dye uptake curve, baseline corrected. Error bars represent S.E.M, n=3; * denotes P<0.05 by one-way ANOVA (Dunnett’s post-test). Bar charts displaying data from 3 separate experiments. (C) Intracellular Ca$^{2+}$ responses were measured in Fura-2 AM loaded J774 cells. Baseline values were recorded for 15 s and then ATP was applied. Fura-2 fluorescence was measured for 300 seconds. An increase in Fura-2 fluorescence was observed on addition of ATP (black circles) in comparison to buffer (light grey diamonds) where no change in fluorescence was seen. P2X7 antagonist AZ10606120 inhibited the response (grey triangles). (D) Data was quantified as mean of sustained Ca$^{2+}$ response (100-300 seconds), baseline corrected. n=3 *denotes P<0.05, significant effect of ATP; one-way ANOVA with Dunnett's post-test.
5.4.2 CK and Rd enhance P2X7 mediated Ca\(^{2+}\) influx in J774 macrophages

Selected ginsenosides were tested for their effect on P2X7 mediated Ca\(^{2+}\) influx with Fura-2 AM loaded cells. It was observed that Ca\(^{2+}\) influx was 186.90 % more in J774 macrophages treated with ATP + 10 µM CK than with ATP alone. In the presence of Rd, Ca\(^{2+}\) influx was increased by 63.17 %. Thus, CK and Rd were able to potentiate P2X7 mediated Ca\(^{2+}\) influx in J774 macrophages (Figure 5.2) similar to HEK-P2X7 cells (Figure 4.6). However, unlike the HEK-293 system, ginsenosides Rb1 and Rh2 did not allow the Ca\(^{2+}\) influx in J774 macrophages. This could be due to a number of factors including the presence of other purinergic receptors in macrophages. From Chapter 4, it was established that ginsenosides are not selective towards P2X7 as they affect P2X4 as well. In addition, the rank order for P2X7 potentiation is HEK-293 cells was CK > Rd > Rb1 > Rh2. Therefore, depending upon the number of P2X7 receptors expressed in a particular cell line, the P2X4-P2X7 interaction in these cells, ginsenoside Rb1 and Rh2 may be unable to replicate the responses in J774 macrophages in comparison to the HEK-293 system where these receptors are over-expressed.

5.4.3 Potentiation of P2X7 mediated YOPRO-1 uptake and Ca\(^{2+}\) influx by CK increases in a concentration dependent manner

The effect of 10 µM CK was tested on YOPRO-1 uptake and Ca\(^{2+}\) influx in J774 macrophages activated at different concentrations of ATP. For dye uptake, ATP concentrations from 10 µM to 1 mM were tested and it was found that the EC\(_{50}\) of ATP for P2X7 was 197.50 µM. In the presence of CK, the EC\(_{50}\) of ATP was reduced to 69.8 µM (95 % confidence interval 47.7 to 102). Therefore, CK lowers the concentration of ATP required to activate P2X7 (Figure 5.3). Measurements of Ca\(^{2+}\) using Fura-2 AM calcium indicator was done in the presence of 10 µM CK and different concentrations of ATP (5µM to 1.5 mM) (Figure 5.4). EC\(_{50}\) of ATP for P2X7 activation was found to be 163.2 µM (95 % confidence interval 92 to 287). In the presence of 10 µM CK, the EC\(_{50}\) values was 109.2 µM (95 % confidence interval 92 to 131).
Various concentrations of CK (10 µM to 1 µM) were tested on J774 macrophages activated by 200 µM of ATP (Figure 5.5). An increase in Ca\(^{2+}\) influx was observed with the increasing concentration of CK. The EC\(_{50}\) of CK for P2X7 activation was found to be 2.2 µM (95% confidence interval 1.2 to 4.5). Therefore, the results obtained for J774 macrophages validate the data from the HEK-293 system (Chapter 4) that CK allosterically modulates P2X7 ion channels.
Figure 5.2 Selected ginsenosides enhance the sustained Ca\textsuperscript{2+} response associated with P2X7 receptor activation in J774 macrophages

(A) Intracellular Ca\textsuperscript{2+} responses were measured in Fura-2AM loaded J774 cells. Baseline values were recorded for 15 s and then ATP was applied. Increase in fluorescence in response to 200 µM ATP represented here in black dots while the potentiation of ATP responses in presence of 10 µM CK shown in red. ATP responses were inhibited by P2X7 antagonist AZ10606120 (shown here in grey). (B) Changes in Fura-2 fluorescence responses were quantified as mean of Fura-2 fluorescence between 100 and 300 s. Baseline correction was done by taking away minimum responses measured from 0-15 seconds mean sustained responses. The Ca\textsuperscript{2+} influx in the presence of 200 µM ATP is shown in solid black bar. The Ca\textsuperscript{2+} influx in ATP activated J77 macrophages is inhibited by 10 µM AZ10606120 (grey open bar). Ca\textsuperscript{2+} influx in the presence of ATP + 10 µM CK is shown as red patterned bar, in the presence of ATP + 10 µM Rd is shown in blue, ATP + 10 µM Rb1 is shown in green and ATP + 10 µM Rh2 is shown in green. The aglycone PPD is shown in green open bar. n=3 *P<0.05, significant effect of ATP; one-way ANOVA with Dunnett's post-test.
P2X7 mediated dye uptake was measured in J774 macrophages activated at different concentrations of ATP (10 µM-1 mM) in the presence and absence of 10 µM CK. YOPRO-1 uptake in presence of ATP alone is shown in black while ATP+ CK responses are shown in red. EC₅₀ of ATP is decreased from 197.50 µM to 69.83 µM (95% confidence interval of 47.5 to 102.2) in the presence of CK. The data was taken from 3 independent experiments and transformed data was been fitted using non-linear fit of regression. The error bars represent S.E.M.
Figure 5.4 Effect of CK on Ca^{2+} influx in ATP activated P2X7 responses in J774 macrophages

Various concentrations of ATP (2 mM- 10 µM) were used to activate the P2X7 receptor and the activation was measured by sustained Ca^{2+} response (Fura-2 fluorescence) on Flexstation 3. CK increased the potency and efficacy of ATP binding to P2X7 in macrophages. The EC_{50} of ATP was lowered from 163.2 µM to 109.2 µM (95 % confidence interval 91.5 to 131.1) in the presence of 10 µM CK. The data was taken from 3 independent experiments and transformed data was fitted using non-linear fit of regression. The error bars represent S.E.M.
Figure 5.5 Effect of CK on ATP-induced Ca\textsuperscript{2+} influx in P2X7 responses in J774 macrophages

Different concentrations (0.1-10 µM) of CK were tested for their effect on Ca\textsuperscript{2+} influx in J774 macrophages stimulated at fixed concentration (200 µM) of ATP. The EC\textsubscript{50} of CK was found to be 2.2 µM (95% confidence interval 1.2 to 4.5). Data was taken from 2 independent experiments (10-12 well). Error bars represent S.E.M.
5.4.4 LPS does not affect P2X7 channel activity in J774 macrophages

During infections, bacterial endotoxin LPS is secreted by bacteria and acts as the greatest stimulus for macrophage activation (489). LPS induced infection can also increase the amount of ATP released in the extracellular matrix, causing activation of P2X7.

Macrophages are known to have a number of LPS binding proteins on their surface including CD14, CD11b, scavenger receptors and toll like receptors (490-493). Due to the absence of a transmembrane domain, CD14 is thought to be unlikely to initiate an LPS response on its own. Evidence from various studies suggests that TLR4s are the most important protein involved in LPS actions. On binding to TLR4, LPS leads to assembly of the NLRP3 inflammasome, which catalyses the conversion of pro-IL1β to mature IL-1β. LPS is also known to directly interact with P2X7. P2X7 has a LPS binding site on its cytoplasmic tail, indicating the possibility of a direct interaction between the two molecules. Extracellular ATP increases during inflammation and injury, leading to activation of P2X7. It has been observed that Ca²⁺ influx and K⁺ efflux associated with P2X7 activation are both essential for NLRP3 inflammasome assembly and is also responsible for mitochondrial ROS secretion upstream of NLRP3 activation (494). P2X7 receptor activation is known to amplify LPS-induced signalling via interleukin-1β release (183, 495). Keeping in mind that LPS/ P2X7 interaction can cause an increased pro-inflammatory response in macrophages, the next step in this study was to find out the effect of ginsenosides, the positive modulators of P2X7 on LPS primed cells. To determine if LPS altered ATP induced responses in J774 cells, the difference in Ca²⁺ influx was measured for LPS primed and non-primed Fura 2-AM loaded J774 macrophages. Again, it was seen that LPS treatment did not influence the influx of Ca²⁺ ion through activated P2X7 channel (Figure 5.6). Ginsenosides were now tested for the difference in their P2X7 potentiating effect in LPS primed and un-primed cells. Similar to observations made in Figure 5.7, only CK and Rd were able to potentiate P2X7 mediated Ca²⁺ entry but there was no difference in the magnitude of
this potentiation between LPS primed and non-primed cells. AZ10606120 (10 µM) inhibited these responses to an equal extent in both the scenarios. From this result, it could be suggested that LPS did not alter the P2X7 channel activity directly.
Figure 5.6 LPS priming of J774 macrophages for 4 hours has no effect on P2X7 mediated Ca\(^{2+}\) responses.

(A) Incubation of J774 macrophages with 100 ng/ml LPS for 4 hours of ATP (200 µM) activation did not have any effect on magnitude of Ca\(^{2+}\) responses as evident from raw traces (baseline corrected) of Fura-2 fluorescence. The Ca\(^{2+}\) responses for LPS primed cells are shown in orange while Ca\(^{2+}\) responses for non-primed macrophages are shown in black. (B) Data was quantified as mean of sustained response from 100-300 seconds. Baseline correction was done by taking away minimum responses measured from 0-15 seconds mean sustained responses. Sustained Ca\(^{2+}\) influx in non-LPS primed macrophages are shown as black bar, Ca\(^{2+}\) influx after ATP activation in LPS primed cells is shown as orange patterned bars. In both cases, P2X7 antagonist, AZ10606120, inhibited the Ca\(^{2+}\) responses to an equal extent. n=3, no significant effect of LPS; one-way ANOVA with Dunnett's post-test.
Figure 5.7 Effect of selected ginsenosides on Ca\(^{2+}\) influx in LPS primed J774 macrophages

(A) Intracellular Ca\(^{2+}\) concentration after ATP activation of P2X7 receptors was observed to be potentiated by ginsenosides CK. The magnitude of this potentiation was unaltered by LPS priming of cells prior to ATP activation. Ca\(^{2+}\) responses of LPS primed cells are shown in orange and those for non-primed cells are shown in black. The data was measured as change in Fura-2 fluorescence on Flexstation 3 (Molecular Devices) (B) Data was quantified as mean of sustained response from 100-300 seconds. Baseline correction was done by taking away minimum responses measured from 0-15 seconds mean sustained responses. The Ca\(^{2+}\) influx was significantly potentiated in presence of ginsenoside CK and Rd but there was no difference in the degree of this potentiation in cells pre-exposed to LPS in comparison to cells. Ginsenosides Rb2, Rh2 and PPD1 do not exhibit a significant change in Ca\(^{2+}\) influx after ATP activation. Sustained responses in presence of without LPS priming is shown as black dotted bar. Ca\(^{2+}\) responses in ATP activated LPS primed cells is shown as orange patterned bar. In both cases, P2X7 antagonist, AZ10606120, inhibits the Ca\(^{2+}\) responses to an equal extent. n=3, * denotes P<0.05, significant effect of ATP; one-way ANOVA with Dunnett’s post-test.
5.4.5 CK and Rd increase the ATP induced cell death

P2X7 is referred to as the “death receptor” as its activation via extracellular ATP following tissue injury or infection leads to switching on of apoptotic cell death pathways (262). There is evidence in support of the role of P2X7 in cell death (496-498), so ginsenosides were tested on their effect on P2X7 mediated cell viability using MTS assay. P2X7 receptor activation requires relatively high concentrations of ATP. By incubating J774 macrophages with various concentrations (ranging from 10 µM – 3 mM) of ATP for 24 hours and then testing the cell viability using tetrazolium salt MTS, it was determined that 3mM ATP was a lethal dose for these cells as it caused more than 70 % reduction in cell viability. ATP (500 µM) was found to be the highest non-lethal dose of ATP. The effect is P2X7 mediated as the P2X7 antagonist AZ10660120 (10 µM) reverts the cell viability to 80 %. So, for testing the effect of ginsenosides on cell viability, the cells were incubated for 24 hours with 10 µM selected ginsenosides in the presence of 500 µM of ATP. It was observed that 10 µM CK in the presence of 500 µM ATP decreased the cell viability of J774 macrophages. The percentage of cell death observed in the presence of 500 µM ATP + 10 µM CK was found to be equivalent to the percentage of cell death observed in the presence of 3 mM ATP (app. 30 %) (Figure 5.8 A). The same effect was not observed with any of the other ginsenosides tested. Therefore, it could be suggested that ginseng metabolite CK was able to enhance the ability of ATP to cause cell death in J774 mouse macrophages. The ability of ginsenosides to increase cell death was then tested in LPS primed cells (Figure 5.8 B). J774 macrophage cells were incubated with 100 ng/ml LPS for 24 hours along with all other treatments. It was observed that 3 mM ATP reduced cell viability to 30% of the untreated controls (Figure 3.8). The effect was P2X7 mediated as the P2X7 antagonist AZ10660120 (10 µM) reverted the cell viability to 81 %. 500 µM ATP was used as a non-lethal dose of ATP as it did not affect cell viability. In the presence of 10 µM CK at 500 µM ATP in LPS treated macrophages, the cells death became equivalent to 3 mM ATP (app. 32 %). The same effect is not observed at lower concentrations of CK. Thus,
in the case of LPS treated cells, CK replicated its ability to enhance cell death in macrophages. Ginsenoside Rd which did not have any cell death enhancing effect in non LPS primed cells caused a decrease in cell viability in the presence of ATP. Rb1 and Rh2 did not show any effect on cell viability in the presence or absence of LPS.
Figure 5.8 Effect of LPS priming on cell viability in ATP stimulated J774 macrophages in the presence of ginsenosides

(A) Cell viability was measured using Cell Titre Aqueous ONE solution (Promega) after incubation J774 macrophage cells with specific treatments for 24 hours (without LPS priming). Cell viability in the presence of ATP is shown in dark grey bars, ATP + 10 µM ginsenosides in black patterned bars and ATP + ginsenoside + AZ10660120 shown in grey patterned bars. Error bars represent SEM, * denotes P<0.05 by one-way ANOVA (Tukey’s multiple comparison test). (B) Cell viability measured after incubation of J774 macrophage cells in the presence of 100 ng/ml LPS with all the other treatments specified in panel A for 24 hours. Cell viability measured in presence of LPS is shown in black patterned bars, LPS + ATP is shown in orange and LPS + ATP + AZ10660120 shown in grey patterned bars. Error bars represent SEM, * denotes P<0.05 by two-way ANOVA (Tukey’s multiple comparison test).
5.4.6 CK and Rd increase P2X7 mediated cellular ROS production

ROS are considered as the key signalling molecules for inflammatory disorders (499). It is known that P2X7 activation can cause production of ROS upstream of the inflammasome assembly (500). The effects of ginsenosides on cellular ROS production were tested by using CM-H2DCFDA dye. It was observed during the optimisation process that if the macrophages were incubated with the CM-H2DCFDA ROS indicator prior to adding the drugs (as suggested by the manufacturer’s instructions), the differences in ROS species were difficult to measure (as ROS are very short lived).

Therefore, for these experiments, the cells were not incubated with CM-H2DCFDA dye before to adding drugs or LPS. The media was removed from the cells and replaced with ROS indicator dye ± drugs. The changes in fluorescence were measured every 15 minutes for up to 2 hours on a Clariostar plate reader. The data were then quantified as fold change from initial to final reading (Figure 5.9). Consistent with the Ca2+ and cell viability data, in ATP activated non LPS-primed J774 macrophages (CM-H2DCFDA fluorescence fold increase- 1.5), ginsenosides CK and Rd increased cellular ROS fluorescence by 11.5 and 7.6 fold respectively (Figure 5.10). In LPS primed cells, it was observed that the ROS production was more in LPS + ATP treated cells (the value being 5.2-fold) in comparison to ATP treated cells only. Ginsenosides CK and Rd again increased these values to 17.8 and 13.5-fold respectively. Ginsenosides Rb1 and Rh2 do not show any significant difference in ROS production (Figure 5.11). Thus, from these results it can be suggested that the LPS priming of J774 macrophages could be causing a greater increase in disruption of the mitochondrial membrane potential in comparison to ATP treated cells alone. This could lead to an increase in production of cellular reactive species, causing an enhancement in the pro-inflammatory properties of ginsenoside CK and Rd.
Figure 5.9 Optimisation of CM-H$_2$DCFDA assay with H$_2$O$_2$ as positive control in the presence and absence of LPS priming in J774 macrophages for measurement of cellular ROS

(A) Cellular ROS production was measured in J774 macrophages as change in CM-H$_2$DCFDA fluorescence for every 15 minutes up to 90 minutes. Amount of cellular ROS produced over time by J774 macrophages in the presence of H$_2$O$_2$ (positive control) is shown in grey circles, LPS + H$_2$O$_2$ responses are shown in orange solid circles, basal responses in presence of buffer are shown in grey triangles and ROS production in presence of LPS (100 ng/ml) are shown as orange open circles. ROS production in LPS primed cells in presence of H$_2$O$_2$ is shown in orange. (B) The data was quantified as fold change in CM-H$_2$DCFDA fluorescence from 0 to 90 minutes. Fold change for buffer shown in grey open bar, for 100 ng/ml LPS shown in orange open bar, 0.01 % H$_2$O$_2$ shown in grey solid bar and H$_2$O$_2$+ LPS shown in orange and grey patterned bar. Error bars represent S.E.M, *$P<0.05$ by one-way ANOVA (Dunnett’s multiple comparison test).
Figure 5.10 Effect of selected ginsenosides on cellular ROS production in the presence and absence of LPS priming in J774 macrophages in CM-H$_2$DCFDA assay.

(A) Ginsenosides CK, Rd, Rb1 and Rh2 were tested for their effect on P2X7 mediated cellular ROS production using CM-H$_2$DCFDA ROS sensitive dye. Increase in CM-H$_2$DCFDA fluorescence over time in the presence of P2X7 agonist ATP ± ginsenosides is shown here as fluorescence (random fluorescence units) at different time points. (B) Data was quantified as fold change in CM-H$_2$DCFDA fluorescence from 0 to 90 minutes. Data from ATP treated controls are represented as grey bars with black outlines, ATP + ginsenosides treated cells are represented as black chequered bars and the ATP + ginsenosides+ AZ10606120 is represented as grey patterned bars. n=3, error bars represent S.E.M, * denotes $P<0.05$; two-way ANOVA with Tukey's post-test.
Figure 5.11 Effect of ginsenosides on cellular ROS production in the presence and absence of LPS priming in J774 macrophages.

(A) Ginsenosides CK, Rd, Rb1, Rh2 and control a glycone PPD1 were tested for their effect on P2X7 mediated cellular ROS production in the presence of 100 ng/ml LPS in J774 macrophages. Increase in CM-H$_2$DCFDA fluorescence over time in the presence of LPS + ATP is shown in orange solid circles, in presence of LPS + ATP + CK is shown in orange open circles. (B) Data was quantified as fold change in CM-H$_2$DCFDA fluorescence from 0 to 90 minutes. Data from LPS + ATP treated controls are represented as white patterned bars with black outlines, LPS + ATP + ginsenosides treated cells are represented as orange chequered bars and the LPS + ATP + ginsenosides + AZ10606120 is represented as grey patterned bars. n=3, error bars represent S.E.M, * denotes P<0.05; two-way ANOVA with Tukey’s post-test.
5.4.7 CK and Rd increase P2X7 mediated mitochondrial superoxide (mtSOX) production

In the cellular systems, there are a number of professional producers of ROS such as NADPH oxidase (NOX), mitochondria, endoplasmic reticulum and peroxisomes. Mitochondria have been considered as the chief source of endogenous ROS in living organisms. After establishing the effect of selected ginsenosides on levels of cellular ROS in J774 macrophages, the effect was reaffirmed using a mitochondria ROS indicator MitoSOX. CM-H$_2$DCFDA used to measure cellular ROS is a general ROS indicator, which measures all endogenous ROS (hydroxal, peroxy and other reactive species) (Figure 5.9 – Figure 5.11). MitoSOX on the other hand, measures superoxide radicals produce in the mitochondria. For measuring changed in MitoSOX fluorescence, the cells were incubated with the dye and drugs all at once and the fluorescence was measured every 15 minutes up to 2 hours (similar to CM-H$_2$DCFDA assay). Actinomycin A and FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydrazone) were used as positive controls. Actinomycin A inhibits the respiratory chain at complex 3 (between cytochrome b and cytochrome c1), thereby preventing the oxidation of both NADH and succinate (501). FCCP, on the other hand, is a respiratory uncoupler which increase proton permeability of the cell and specifically uncouples electron transport chain from ATP synthesis (502) Both compounds increase the production ROS from mitochondria, so they are suitable positive controls for the MitoSOX assay. Both antimycin A (10 µM) and FCCP (5 µM) showed a consistent increase in ROS production over the period of measurements (Figure 5.12). The results from the mitochondria superoxide measurements confirmed that ginsenoside CK and Rd increased the superoxide production by acting on ATP activated P2X7 in macrophages. The effect was observed to be enhanced in presence of LPS. These results confirmed that CK and Rd but not Rb1 and Rh2 increased the ROS mainly from mitochondrial source. It was further observed that LPS enhanced the effect of ATP mediated production of mitochondrial
superoxide (mtSOX) as well as increased the ability of ginsenoside CK and Rd to accentuate ATP mediated mtSOX production. Macrophages are known to produce ROS by various mechanisms such as mitochondrial damage, NADPH oxidases (NOXs) and endoplasmic reticulum stress. NADPH oxidase is a membrane bound complex which is responsible for production of superoxide and H$_2$O$_2$. Recent studies have indicated the involvement of NOX2 in the production of mitochondrial superoxide. Thus, the increased production of mitochondrial superoxide on the exposure of macrophages to LPS (Figure 5.14) could be due to the involvement of NOX2. In presence of LPS, ATP treated macrophages had 5.2-fold change in MitoSOX fluorescence, LPS + CK + ATP had a 17.83 fold change (6.31 more fold change that non-LPS treated macrophages); LPS + Rd + ATP increased ROS production by 13.54 fold (5.91 more fold change than non-LPS treated cells). P2X7 inhibitor AZ10660120 (10 µM) was able to inhibit this increase in all treatments affirming that these responses are P2X7 mediated.
Figure 5.12 Effect of ginsenosides on mitochondrial superoxide (mtSOX) productions in the presence of FCCP and Actinomycin in ATP activated J774 macrophages.

(A) mtSOX production was measured in ATP activated J774 macrophages every 15 minutes for 90 minutes. FCCP and Actinomycin A were used as positive controls. Changes in MitoSOX fluorescence in presence of buffer are shown in grey dots. An increase in ROS production was seen in presence of 10 µM Actinomycin A (blue dots). (B) Data was quantified as fold change in fluorescence between time points 0 to end point of 90 minutes. Grey bar represents ROS production by macrophages in buffer, black patterned bar represents data from 500 µM ATP, positive controls FCCP and Antimycin A are represented by cyan coloured bar and open blue bar respectively. n=3, error bars represent S.E.M, * P<0.05; one-way ANOVA with Dunnett’s multiple comparison test.
Figure 5.13 Effect of ginsenosides on mitochondrial superoxide productions in ATP activated J774 macrophages.

(A) Ginsenosides CK, Rd, Rb1 and Rh2 were tested for their effect on P2X7 mediated mtSOX production. Increase in MitoSOX fluorescence from 0 to 105 minutes in the presence of ATP are shown in black solid circles and MtSOX production in presence of CK+ ATP is shown in black open circles. (B) Data was quantified as fold change in MitoSOX fluorescence (by deducting initial value from final value and the dividing by initial fluorescence value). ATP+ ginsenosides treated controls are represented as black patterned bars; ATP+ ginsenosides + AZ10606120 treated cells are represented as grey pattered bars. n=3, error bars represent S.E.M, *represents P<0.05, two-way ANOVA with Tukey's post-test
Figure 5.14 Effect of ginsenosides on mitochondrial superoxide production in presence of 100 ng/ml LPS in ATP activated J774 macrophages.

(A) Ginsenosides CK, Rd, Rb1 and Rh2 were tested for their effect on P2X7 mediated mtSOX production in the presence of 100 ng/ml LPS. Increase in MitoSOX fluorescence from 0 to 105 minutes in the presence of LPS + ATP is shown in orange solid circles and mtSOX production in presence of LPS + CK + ATP is shown in black open circles. (B) Data was quantified as fold change in MitoSOX fluorescence. LPS + ATP treated controls are represented as orange patterned bars; ATP + ginsenosides treated cells are represented as grey patterned bars and responses from LPS+ATP+ ginsenosides + AZ10606120 are shown in grey open bars, n=3 *P<0.05, two-way ANOVA with Tukey's post-test.
5.8 CK increases caspase 3/7 activity in ATP activated J774 macrophages

The measurement of cell death by MTS assay provided an evidence of extent of cell death but did not establish the mechanism of cell death pathway involved. The progression of cell death involves activation of a number of processes before the cell reaches a point-of-no-return and commits itself to death. The cells on a death route would display a few characteristic features such as large activation of caspases (503), disruption of mitochondrial membrane potential (504), permeabilisation of outer mitochondrial membrane (505) and exposure of phosphatidyl serine. All the above act as important biological responses to initiate phagocytosis (506). P2X7 activation is associated with change in cell morphology including membrane blebbing, phosphatidylserine exposure and activation of classical apoptotic signalling cascade (79, 450, 507). Wen et al. reported the activation of caspase-3 and PARP-1 in ATP activated apoptosis in HEK-293 cells expressing P2X7 (508).

In an attempt to establish the signalling molecules that potentiated by CK and Rd via ATP ± LPS activated P2X7 in macrophages, the activity of caspase 3/7 was measured in J774 macrophages. Caspases are the members of the caspase family of proteases that regulate cell death pathways including apoptosis (509). Various cell death stimuli such as injury, exposure to inflammatory stimuli (such as LPS) or any other insults can result in activation of caspases which, in a systematically coordinated manner, work towards proteolysis of numerous cellular proteins, ultimately leading to cell death. Caspase 3 and 7 along with caspase 9 work downstream in the cell death pathways are referred as “effector” caspases. They are known to handle the bulk of proteolysis implicated in cell death (510). P2X7 is known to mediate apoptosis/cell death via activation of caspase 3/7/9 (511)

The cell viability results from MTS assay clearly indicate that CK increases cell death at otherwise non-lethal concentrations of ATP (500 µM), but these results are not indicative of the signalling molecules that are actually switched on during the process. For the purpose of
finding whether caspase 3/7 activity is affected by presence of ginsenosides in ATP-P2X7 axis in macrophages, CellEvent™ Caspase-3/7 Green Detection Reagent was employed along with NucBlue™ Live ReadyProbes™ Reagent (for cell number). The caspase 3/7 activity was observed in live cells by capturing images every 30 minutes for a 12 hours period, using Image Express (Molecular Devices). Caspase activity was recorded as green fluorescence and the number of cells was counted on the basis of blue fluorescence from NucBlue™ Live ReadyProbes™ Reagent. Images taken every 30 minutes were later analysed using MetaExpress software (Figure 5.16 and Figure 5.18). The data was expressed as percentage of caspase 3/7 positive cells. 0.5 µM staurosporine (STR) was used a positive control as STR is known to induce apoptosis by activating caspases and is a recommended positive indicator of apoptotic cell death (512). It was observed that a robust increase in caspase 3/7 activity took place in presence of STR in comparison to control cells (only buffer) (Figure 5.15). 500 µM ATP caused a similar increase in caspase 3/7 and most of the response was inhibited by P2X7 antagonist AZ10606120, confirming that it was a P2X7 mediated effect. Various concentrations of CK (1, 5, 10 and 20 µM) were tested in order to ascertain the most optimum concentration for measuring this activity. The criterion for optimum concentration was choosing a concentration, which was non-toxic on its own and produced a robust, measurable caspase 3/7 response. It was found that at 20 µM concentration of CK, 100 % cells were caspase 3/7 positive within the first 2 hours of the measurement (Figure 5.15). This was a P2X7 independent effect as AZ10606120 was unable to inhibit the caspase 3/7 activity at this concentration of CK. All concentrations below 10 µM CK was non-toxic on their own. Therefore, 10 µM was chosen to be the test dose. Since 20 µM CK gave immediate and very strong caspase activity signals (with about 93 % of the cells showing caspase 3/7 activity at time point 6), it was decided to include 20 µM CK as a positive control for all these experiments. Ginsenosides CK, Rd, Rb1 and Rh2 at a concentration of 10 µM were tested for their effect on caspase 3/7 activity (Figure 5.17). It was seen that 500 µM ATP on its own took
6 hours to initiate a caspase 3/7 activity and at the end of the 12 hours measurement period, 50% of the macrophages were caspase 3/7 active. Ginsenosides CK, Rd, Rb1 and Rh2 at a concentration of 10 µM were then tested and it was found that in the presence of CK + ATP, at the end of the time point 6 (3 hours) 88% of J774 macrophages were positive. At the end of the measurements (12 hours), caspase 3/7 were active in 100% of the macrophages. P2X7 antagonist AZ10606120 was able to inhibit the caspase 3/7 activity, indicating that P2X7 was involved in activation of these caspases in the cells. Rd, Rb1 and Rh2 did not show any potentiation of ATP- P2X7 mediated caspase activity. Even though all these ginsenosides elicited high caspase 3/7 activity at 20 µM concentration (in the absence of ATP- in a P2X7 independent manner), at 10 µM concentrations only CK was able to elevate the ATP- P2X7 mediated increase in caspase 3/7 activity. These results indicate that CK increase the pro-inflammatory ability of ATP by increasing Ca\textsuperscript{2+} / K\textsuperscript{+} ion fluxes, increasing ROS production and resulting in a positive effect on caspase 3/7 activity in J774 macrophages. A similar trend was seen with control 0.01 % DMSO where approximately 50% of the cells were positive for caspase activity.
Figure 5.15 Measurement of caspase 3/7 activity in the presence of apoptotic inducer staurosporine for optimisation of assay

(A) The graph shows the fluorescence of CellEvent™ Caspase-3/7 Green Detection Reagent for each time point measured (every 30 minutes for 12 hours). The basal caspase 3/7 activity indicated by increase in green fluorescence over time in J774 macrophages treated with 0.01 % DMSO is shown in grey circles, caspase 3/7 activity in presence of P2X7 agonist ATP is shown in black. Caspase 3/7 activity in presence of staurosporine (STR, used here as positive control for apoptosis) is shown in grey circles with black outlines and finally responses in presence of 20 µM CK is shown in red open circles. (B) The percentage J774 macrophages positive for Caspase-3/7 activity at time point 6 of the assays (time point 12; 6 hours) were then plotted as a bar graph. 500 µM ATP is shown as black solid bar, 20µM CK as red open bar, 0.5µM STR as red patterned bar and 0.01% DMSO as black open bar. The percentage positive cells in presence STR are significantly higher than those in presence of ATP on its own. n=3 *P<0.05, significant effect of 0.5 µM STR and 20 µM CK ; one-way ANOVA with Dunnet’s multiple comparison test.
Figure 5.16 NucView 488 3/7 caspase activity in presence of 0.01% DMSO, 0.5 µM STR and 20 µM CK

Representative images of caspase 3/7 activity of J774 macrophages in presence of 0.01% DMSO, 0.5 µM STR taken at time point 12 (after 6 hours) are shown here. The images were taken under three filters (A) Images taken under transmitted light showed the changes in morphology of the macrophages through time. The cells were adherent to the surface till the end of the assay. Some cellular debris could be observed, and the cell membranes started to look shrivelled for cells treated with 20 µM CK. (B) The NucBlue dye stained the nucleus and was used to assist in keeping track of the cell number. The cell number represented by blue fluorescence remained constant throughout the assay. The cell membrane showed some fragmentation, but the nuclei remained stained. (C) Green fluorescence indicated caspase 3/7 activity. It can be seen that in J774 macrophages treated with 0.01% DMSO, there is negligible caspase 3/7 activity at the end of 6 h incubation whereas in macrophages treated with 0.5 µM STR, approximately 70% cells showed green fluorescence while in presence of 20 µM CK, around 95% cells were positive of caspase 3/7 activity.
Figure 5.17 Measurement of caspase 3/7 activity in the presence of selected ginsenosides CK, Rd, Rb1 and Rh2

(A) The graph shows the fluorescence of CellEvent™ Caspase-3/7 Green Detection Reagent for each time point measured (every 30 minutes for 12 hours). Percentage of macrophages positive for caspase 3/7 activity (0-12 hours, recorded every 30 minutes) in presence of 500 µM ATP is shown in black circles, caspase activity in presence of ATP + CK is shown in red circles, ATP + Rd in blue squares, ATP + Rb1 shown in green triangles and ATP + Rh2 is shown in purple inverted triangles. (B) The percentage J774 macrophages positive for caspase 3/7 activity at time point 12 of the assays (time point 12; 6 hours) were then plotted as a bar graph. 500 µM ATP is shown as black solid bar, ATP + 10 µM CK as red patterned bar, ATP + 10 µM Rd as blue patterned bar, ATP + 10 µM Rb1 as green patterned bar and ATP + 10 µM Rh2 as purple patterned bar as black open bar. The percentage positive cells in presence ATP+CK are significantly higher than those in presence of ATP on its own. n=3, error bars represent S.E.M, * denotes P<0.05, one-way ANOVA with Dunnet’s multiple comparison test.
Figure 5.18 NucView 488 3/7 caspase activity in presence of 500 µM ATP, ATP + CK and ATP + CK + AZ10606120

Representative images of caspase 3/7 activity of J774 macrophages in presence of 500 µM ATP, 500 µM ATP + CK and 500 µM ATP + CK + AZ10606120 taken at time point 12 (after 6 hours). The images were taken under three filters (A) Images taken under transmitted light show the changes in morphology of the macrophages through time. The cells are adherent to the surface till the end of the assay. Some cellular debris could be observed, and the cell membranes started to look shrivelled for cells treated with ATP + 10 µM CK. (B) The NucBlue dye stained the nucleus and was used to assists in keeping track of the cell number. The cell number represented by blue fluorescence remained constant throughout the assay. The cell membrane showed some breakdown, but the nuclei are stained. (C) Green fluorescence indicates caspase 3/7 activity. It can be seen that in J774 macrophages treated with 500 µM ATP, there was negligible caspase 3/7 activity at the end of 6 h incubation whereas in macrophages treated with 500 µM ATP + CK, approximately 92 % cells show green fluorescence.
5.4.9 CK and Rd increase caspase 3/7 activity in the presence of LPS in ATP activated macrophages

LPS primed J774 macrophages were then tested to determine whether caspase 3/7 activation kinetics were altered. It was found that LPS caused an increase in the baseline caspase 3/7 activity with more LPS treated cells being activated in comparison to controls from the beginning of the assay (Figure 5.19). There was an increase in overall caspase 3/7 activity in all the conditions tested. 10 µM CK replicated its behaviour in the presence of 500 µM ATP and 100 ng/ml LPS achieving the highest caspase activity at about time point 6 (after 3 hours) with 100 % cells showing positive for caspase 3/7 from this point onward. Rd, Rb1 and Rh2 did not cause a significant increase in caspase activity in presence of LPS + ATP (Figure 5.20). In comparison to LPS non-treated cells, more caspase 3/7 activity was observed when Rd, Rb1 or Rh2 was added to LPS treated macrophages (Figure 5.19). The data from cell viability and caspase 3/7 measurement is indicative of the involvement of ginsenosides CK in potentiating the pro-inflammatory potential of P2X7 in macrophages confronted with inflammatory stimuli, which may coerce the cells towards apoptotic cell death pathways. The clear evidence for the cell death pathway followed by the activation is not precisely evident from these results so further validation using TUNEL or DNA fragmentation could be employed.
Figure 5.19 Measurement of caspase 3/7 activity in the presence of selected ginsenosides CK, Rd, Rb1 and Rh2 in LPS primed J774 macrophages.

(A) The graph shows the fluorescence of CellEvent™ Caspase-3/7 Green Detection Reagent for each time point measured (every 30 minutes for 12 hours). Data is represented as percentage of macrophages positive for caspase 3/7 activity (0 - 12 hours, recorded every 30 minutes). (B) The percentage J774 macrophages positive for caspase-3/7 activity at time point 12 of the assay (6 hours) were then plotted as a bar graph. The percentage positive cells in presence LPS + 0.5 µM STR, LPS + 20 µM CK and LPS + 500 µM ATP are significantly higher than those in presence of 100 ng/ml LPS on its own. n=3, error bars represent S.E.M, *denotes P<0.05, one-way ANOVA with Dunnet’s multiple comparison test.
Figure 5.20 Measurement of caspase 3/7 activity in the presence of selected ginsenosides CK, Rd, Rb1 and Rh2 in LPS primed J774 macrophages.

(A) The graph shows the fluorescence of CellEvent™ Caspase 3/7 Green Detection Reagent for each time point measured (every 30 minutes for 12 hours). Data is represented as percentage of macrophages positive for caspase 3/7 activity (0-12 hours, recorded every 30 minutes). (B) The percentage of J774 macrophage cells positive for caspase 3/7 activity at time point 12 of the assays (6 hours) were then plotted as a bar graph. The percentage positive cells in presence LPS+ ATP + CK are significantly higher than those in presence of LPS + ATP on its own. n=3 * denotes $P<0.05$, one-way ANOVA with Dunnet’s multiple comparison test.
5.4.10 Effect of selected ginsenosides on P2X7 mediated responses in primary peritoneal macrophages

The J774 macrophage cell line is a well-characterised and reliable model for immunological studies (481). J774 is also frequently used because it is readily available, and its use reduces the number of animals involved in research. Conversely, there are some studies indicating that responses measured using J774 macrophages may be weaker to or different from those of primary macrophages (513). Methods involving isolation and maintenance of peritoneal macrophages in cultures have been previously well defined. It is also known that P2X7 receptor is endogenously expressed in these cells. Therefore, some of the experiments performed using J774 macrophages in this study were also validated further using primary peritoneal macrophages (Figures 5.21, 5.22, 5.23, 5.24 and 5.25). The cells were isolated from the peritoneum of Wistar rats.

5.4.11 CK and Rd increase Ca\(^{2+}\) influx in ATP activated primary peritoneal macrophages

In consensus with the data obtained using the J774 macrophage cell line, it was observed that CK and Rd significantly increased the concentration of intracellular Ca\(^{2+}\) following activation of P2X7 by ATP (Figure 5.21). The responses were inhibited by P2X7 antagonist AZ10606120. The potentiation by 10 µM CK was greater in primary peritoneal macrophages than in the J774 cell line. The magnitude of the data was not significantly different in the two types of cell sources. The results here corroborated our results obtained with the J774 macrophage cell line. The effect of different concentrations (0.1- 50 µM) of CK on Ca\(^{2+}\) influx in primary macrophage cells was tested by stimulating these cells at fixed concentration (200 µM) of ATP. The EC\(_{50}\) of CK was found to be 0.5 µM (95 % confidence interval – 0.2 to 1.0) (Figure 5.22).
5.4.12 Effect of ginsenosides on cell viability in primary peritoneal macrophages

Primary peritoneal macrophages were plated in 96 well plates overnight (± drugs, ± LPS) and cell viability was measured using MTS. ATP at 500 µM did not cause any cell death after 24 hours treatment. Instead the cell number was higher in ATP treated cells in comparison to untreated controls. Results from these experiments confirmed that 10 µM CK could accelerate the death in ATP activated macrophages (CK + ATP cause ~ 45 % cell death) in a P2X7 mediated manner. 10 µM Rd, on the other hand, was able to potentiate this cell death only in the presence of pro-inflammatory stimulus 100 ng/ml LPS. The increase in cell death was P2X7 mediated as the effect was reversed in the presence of P2X7 antagonist AZ10606120 (Figure 5.23).

5.4.13 Effect of ginsenosides on mitochondrial superoxide production in primary peritoneal macrophages

The effect of selected ginsenosides was tested on mitochondrial superoxide production. Ginseng metabolite, CK, increased ROS production (superoxide radicals) ± LPS in primary peritoneal macrophages (Figure 5.24). FCCP (carbonilcyanide p-trifluoromethoxyphenylhydrazone), the mitochondrial uncoupler, at a concentration of 5 µM, was used as a positive control for the measurement of mitochondrial superoxides. The other ginsenosides increase in ROS production (± LPS) significantly in comparison to ATP treated controls. LPS priming was observed to cause a general increase in ROS production in all conditions tested.

5.4.14 Effect of CK on caspase 3/7 activity in primary peritoneal macrophages

Primary peritoneal macrophages were also employed for confirming the caspase 3/7 activity data obtained using J774 macrophages. The numbers of peritoneal macrophages available for these experiments were limited, so only the ginsenoside CK, which had consistently shown
potentiation of P2X7 responses, was selected to be tested on primary peritoneal macrophages. The data from these experiments confirmed the ability of 10 µM CK to increase the caspase activity 3/7 (Figure 5.25). The responses are similar in that presence and absence of LPS. In presence of LPS, 4.4 % more peritoneal macrophages were caspase 3/7 positive in comparison to cells treated with ATP only. In presence of 0.5 µM STR (± LPS), ~ 45 % of the cells were caspase 3/7 active and LPS treatment did not cause any significant increase in the caspase activity. Similarly, 10 µM CK treatments resulted in the caspase 3/7 activation in ~ 99 % cells after 6 hours of exposure and these responses were similar both in presence and absence of LPS.
Figure 5.21 Effect of ginsenosides on Ca\(^{2+}\) influx in primary peritoneal macrophages

(A) Intracellular Ca\(^{2+}\) responses were measured in Fura-2AM loaded primary peritoneal macrophages (isolated from peritoneum of Wistar rats). Baseline values were recorded for 15 seconds and then ATP was applied. Fura-2 responses were measured over time period of 300 seconds on Flex station 3. (B) Primary peritoneal macrophages were treated with 200 µM ATP, 200 µM ATP + ginsenosides and 200 µM ATP + 10 µM AZ10606120. Quantitative measures of sustained Ca\(^{2+}\) response (mean fluorescence between 100 to 300 seconds - mean of baseline fluorescence; from 0-15 seconds) was done. The responses are inhibited by P2X7 antagonist AZ10606120 (shown here as blue open bar). Data was collated from 2 independent experiments (6 rats), *P <0.05, significant effect of ATP; one-way ANOVA with Dunnett’s post-test.
Figure 5.22 Effect of CK on ATP-induced Ca$^{2+}$ influx in P2X7 responses in primary peritoneal macrophages

Different concentrations (0.1-50 µM) of CK were tested for their effect on Ca$^{2+}$ influx in primary macrophage cells stimulated at fixed concentration (200 µM) of ATP. The EC$_{50}$ of CK was found to be 0.5 µM (95% confidence interval–0.2 to 1.0). Data is taken from 2 independent experiments (6 rats).
Figure 5.23 Effect of selected ginsenosides on cell viability in primary peritoneal macrophages

(A) Cell viability of rat peritoneal macrophage cells measured using Cell Titre Aqueous ONE solution (Promega) after incubation specific treatments for 24 hours (without LPS). Cell viability of primary peritoneal macrophages in presence of ATP/ATP + ginsenosides is shown in grey solid bars and ATP + ginsenoside + AZ10660120 shown in black patterned bars. Error bars represent S.E.M, * P<0.05 by one-way ANOVA (Tukey’s multiple comparison test). (B) Cell viability measured in J774 macrophage cells in the presence of 100 ng/ml LPS with all the treatments (ATP, ATP + ginsenosides, ATP + ginsenosides + AZ10660120) for 24 hours. Cell viability measured in presence of LPS + ATP / LPS + ATP + ginsenosides is shown in black patterned bars and LPS + ATP + AZ10660120 shown in orange patterned bars. Error bars represent S.E.M, * P<0.05 by one-way ANOVA (Tukey’s multiple comparison test).
Ginsenosides CK, Rd, Rb1 and Rh2 were tested for their effect on P2X7 mediated mtSOX production in primary peritoneal macrophages. The change in MitoSOX fluorescence was measured every 15 minutes for 105 minutes. Data was quantified as fold change in MitoSOX fluorescence. Data from ATP + ginsenosides treated cells are represented as black dotted bars; ATP + ginsenosides + LPS treated cells are represented as orange chequered bars. Data collated from macrophages isolated from 6 rats. Error bars represent S.E.M * denotes $P<0.05$; two-way ANOVA with Tukey's post-test
Figure 5.25 Effect of LPS priming on caspase 3/7 activity in ATP stimulated peritoneal macrophages in the presence of ginsenosides

(A) The graph shows the fluorescence of CellEvent™ Caspase-3/7 Green Detection Reagent for each time point measured (every 30 minutes for 12 hours). Data is represented as percentage of macrophages positive for caspase 3/7 activity (0-12 hours, recorded every 30 minutes). The peritoneal macrophages from Wistar rats were treated with 500 µM ± LPS, 0.5 µM STR ± LPS, 10 µM CK + ATP ± LPS. (D) The percentage of J774 macrophage cells positive for caspase3/7 activity at time point 12 of the assays (6 hours) were then plotted as a bar graph. The percentage positive cells in presence ATP + CK ± LPS are significantly higher than those in presence of LPS + ATP treated cells. The presence of LPS did not alter the caspase 3/7 activity in any of the treatments. n=3 * Error bars represent S.E.M * denotes P<0.05; two-way ANOVA with Tukey’s post-test
5.4.15 Effect of ginsenosides on cytokine secretion in J774 macrophages

To measure the effect of selected ginsenosides on secretion of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6), the J774 macrophages were treated with ginsenoside ± ATP and ± LPS. ELISA was performed to measure the quantity of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) released. In macrophage cells not primed with 100 ng/ml LPS, treatment of cells with ginsenosides did not result in any significant change in cytokine secretion (Figure 5.24 A, Figure 5.25 A and Figure 5.56 A). This observation was consistent with the report that production of proinflammatory cytokines occur in the presence of inflammatory stimuli (such as LPS) was essential in addition to a K+ releasing stimulus such as ATP (514).

In LPS primed cells, LPS treatment alone in the absence of ATP did not cause an increase in cytokine production. This observation was consistent with previous reports that induction of IL-1β maturation and externalisation by TLR agonists (LPS) is very inefficient in the absence of a secondary signal such as extracellular ATP (515-517). It is known that LPS priming is a general prerequisite for activation of caspase-1 in ATP stimulated cells (518).

In the presence of LPS, extracellular ATP caused a significant increase in secretion of cytokines (Figure 5.24 B and Figure 5.25 B). TNF-α is a pleiotropic cytokine which is secreted by many cells especially the ones from monocytic lineage (macrophages, microglia Kupffer cells, Langerhans cells, astroglia) (519). TNF-α is a potent pro-inflammatory mediator that controls several aspects of macrophage function. It is released following injury, infection (exposure to bacterial-derived LPS) and is said to be one of the most abundant early mediators of inflammation (520). Measurements of TNF-α secretion (Figure 5.24) showed the secretion of this cytokine was 51.8 % more in macrophages treated with LPS + ATP + CK in comparison to cells treated with LPS + ATP alone. An increase of 54.8 % in TNF-α secretion was observed in presence of 10 µM Rd+ ATP in LPS primed macrophages. 10 µM Rb1 and Rh2 treatment increased TNF-α by < 40 % (not statistically significant). Figure 5.25 illustrates the secretion
of IL-1β from LPS-primed ATP activated J774 macrophages ± ginsenosides (quantities expressed in pg/mL). It was observed that there was an 82.8% increase in IL-1β in LPS primed ATP activated macrophages treated with 10 µM CK. An increase of 81.6% was observed in the presence of 10 µM Rd and 75.5% in presence of 10 µM Rb1. An increase of 26.6% was observed in presence of 10 µM Rh2 but this enhancement was not statistically significant.

As observed in case of the other two cytokines (TNF-α and IL-1β), ATP activation by ATP alone did not induce IL-6 secretion but LPS priming before ATP activation increased the IL-6 secretion by 92% (Figure 5.26 B). Ginsenosides treatment, on the other hand, did not increase the IL-6 release significantly. CK was seen to cause the maximal increase (~ 20%) among the ginsenoside tested (Figure 5.26 B).
Figure 5.24 TNF-α production by J774 macrophages with and without LPS stimulation

(A) J774 macrophages were incubated with ginsenosides ± 500 µM ATP for 30 minutes. Supernatants were collected and TNF-α was measured by ELISA. The increase in ATP- P2X7 mediated TNF-α secretion by J774 macrophages in presence of ginsenosides was not significant. Error bars represent S.E.M, no significant effect of treatments observed. *P<0.05 Two-way ANOVA, Tukey’s multiple comparison tests.

(B) TNF-α production by LPS-stimulated J774 macrophages was measured by priming the cells with 100 ng/LPS for 4 hours before stimulating these cells with 500 µM ATP ± ginsenosides for 30 minutes. Supernatants were collected precisely after 30 minutes incubation period and TNF-α was measured using ELISA. TNF-α secretion in LPS primed macrophages was significantly increased in presence of 10 µM CK and 10 µM Rd. Statistical analysis was done using Graph Pad prism; Graphs are means (± S.E.M) from 3 independent experiments, and each experiment was performed in duplicates. Error bars represent S.E.M, * denotes *P<0.05 Two-way ANOVA, Tukey’s multiple comparison test.
Figure 5.25 IL-1β productions by J774 macrophages with and without LPS stimulation

(A) J774 macrophages were incubated with ginsenosides ± 500 µM ATP for 30 minutes. Supernatants were collected and IL-1β was measured by ELISA. No significant increase in IL-1β production was observed after ginsenosides treatment in ATP stimulated J774 macrophages. Error bars represent S.E.M. Statistical analysis was done using Graph Pad prism: Two-way ANOVA, Tukey’s multiple comparison test. 

(B) IL-1β production by LPS-stimulated J774 macrophages was measured by priming the cells with 100 ng/LPS for 4 hours before stimulating with 500 µM ATP ± ginsenosides for 30 minutes. Supernatants were collected precisely after 30 minutes incubation period and IL-1β was measured using ELISA. IL-1β secretion in LPS primed J774 macrophages was significantly increased in presence of 10 µM CK and 10 µM Rd. This increase in inhibited by P2X7 antagonist AZ10606120. Graphs are means (± S.E.M) from 3 independent experiments, and each experiment was performed in duplicates; * denotes P<0.05 Two-way ANOVA, Tukey’s multiple comparison test
Figure 5.26 IL-6 production by J774 macrophages with and without LPS stimulation

(A) J774 macrophages were incubated with ginsenosides ± 500 µM ATP for 30 minutes. Supernatants were collected and IL-6 was measured by ELISA. No significant increase in IL-6 production was observed through ginsenosides treatment in ATP stimulated J774 macrophages. Error bars represent S.E.M. Statistical analysis done using Graph Pad prism; Two-way ANOVA, Tukey’s multiple comparison test.

(B) IL-6 production by LPS-stimulated J774 macrophages were measured by priming the cells with 100 ng / LPS for 4 hours before stimulating these cells with 500 µM ATP with or without ginsenosides for 30 minutes. Supernatants were collected precisely after 30 minutes incubation period and IL-6 was measured using ELISA. An increase in IL-6 secretion in LPS primed macrophages in comparison to non-primed macrophages was observed. Selected ginsenosides caused an increase in IL-6 production in LPS primed ATP stimulated J774 macrophages but this increase was not statistically significant. Graphs are means (± S.E.M) from 3 independent experiments, and each experiment was performed in duplicates. ns denotes not significant, Two-way ANOVA, (Tukey’s multiple comparison test).
5.4.16 Effect of ginsenosides on cytokine secretion in primary peritoneal macrophages

In order to confirm the results obtained using J774 macrophages, TNF-α, IL-1β and IL-6 secretions were measured in ATP activated primary peritoneal macrophages (isolated from peritoneum of Wistar rats) with and without LPS priming. Measurements of TNF-α secretion (Figure 5.27) showed that LPS priming before ATP activation increased TNF-α by 60.5 %. An increase of < 35 % in TNF-α secretion was observed in presence of all ginsenosides in LPS primed macrophages but none of this enhancement was statistically significant (Figure 5.27). This is different from the observation made in J774 macrophages (Figure 5.24). Figure 5.28 shows the measurement of IL-1β in primary macrophages. An increase in IL-1β was observed in LPS primed ATP stimulated rat macrophages in the presence of all ginsenosides. Measurement of IL-6 in primary macrophages is shown in Figure 5.29. An increase in IL-6 is observed in LPS primed primary macrophages in the presence of CK. This is different from J774 macrophage cell line, where none of the ginsenoside tested caused any significant change in IL-6 secretion (Figure 5.26).
Figure 5.27 TNF-α production by primary peritoneal macrophages ± LPS stimulation

(A) Primary peritoneal macrophages isolated from Wistar rats were incubated with ginsenosides ± 500 µM ATP for 30 minutes. Supernatants were collected and TNF-α was measured by ELISA. The difference in ATP-P2X7 mediated TNF-α secretion by rat macrophages in presence of ginsenosides was not significant. Statistical analysis done using Graph Pad prism; Graphs are means (± S.E.M) from 6 different rats. Error bars represent S.E.M. Two-way ANOVA, Tukey’s multiple comparison test (B) TNF-α production by LPS-stimulated rat macrophages by measured by priming the cells with 100 ng/LPS for 4 hours before stimulating these cells with 500 µM ATP with or without ginsenosides for 30 minutes. TNF-α secretion in LPS primed macrophages is significantly increase in presence of 10 µM CK and Rd. Statistical analysis done using Graph Pad prism; Graphs are means (± S.E.M) from 6 independent rats. Error bars represent S.E.M * denotes P<0.05 Two-way ANOVA, Tukey’s multiple comparison test.
Figure 5.28 IL-1β production by primary peritoneal macrophages ± LPS stimulation

(A) The peritoneal macrophages isolated from Wistar rats were incubated with ginsenosides and/or 500 µM ATP for 30 minutes. Supernatants were collected and IL-1β was measured by ELISA. The difference in ATP-P2X7 mediated IL-1β secretion by rat macrophages in presence of ginsenosides was not significant. (B) IL-1β production by LPS-stimulated human macrophages by measured by priming the cells with 100 ng/LPS for 4 hours before stimulating these cells with 500 µM ATP with or without ginsenosides for 30 minutes. Supernatants were collected after 30 minutes and IL-1β secretion in LPS primed macrophages is significantly increase in presence of 10 µM CK and Rd. Statistical analysis done using Graph Pad prism; Graphs are means (± S.E.M) from 6 independent rats. Error bars represent S.E.M, *denotes $P<0.05$ Two-way ANOVA, Tukey’s multiple comparison test.
Figure 5.29 IL-6 production by rat macrophages with and without LPS stimulation

(A) The peritoneal macrophages isolated from Wistar rats were incubated with ginsenosides and/or 500 µM ATP for 30 minutes. Supernatants were collected and IL-6 was measured by ELISA. The difference in ATP-P2X7 mediated IL-6 secretion by rat macrophages in presence of ginsenosides was not significant. (B) IL-6 production by LPS-stimulated human macrophages by measured by priming the cells with 100 ng/LPS for 4 hours before stimulating these cells with 500 µM ATP with or without ginsenosides for 30 minutes. Supernatants were collected after 30 minutes and IL-6 was measured using ELISA. IL-6 secretion in LPS primed macrophages is significantly increase in presence of 10 µM CK and Rd. Statistical analysis done using Graph Pad prism; Graphs are means (± S.E.M) from 6 independent rats. Error bars represent S.E.M * P<0.05 Two-way ANOVA, Tukey’s multiple comparison.
5.5 Key results from this study are summarised in Figure 5.30

Figure 5.30 Diagrammatic representations of the experimental findings in the present study.
The key results from this study can be summarised from this illustration are:

- Out of the 4 ginsenosides tested, CK and Rd potentiated P2X7 mediated Ca\(^{2+}\) influx in macrophages (both J774 and peritoneal macrophages).
- LPS priming did not cause any additional increase in P2X7 mediated Ca\(^{2+}\) influx potentiated by ginsenosides CK and Rd.
- Ginseng metabolite CK increased cell death via P2X7 activation. This ability was not exhibited by Rd, Rb1 and Rh2.
- In the presence of LPS, CK and Rd both decreased the viability of J774 macrophages via potentiation of P2X7.
- CK increased caspase 3/7 activity in ATP activated J774 macrophages.
- In presence of LPS, there was a general increase in caspase 3/7 activity by all ginsenosides tested but the increase was not significant statistically. Time required to achieve 100 % activity by CK + ATP was reduced by LPS priming in J774 macrophages.
- In the presence of LPS, CK and Rd increased the cellular ROS and mitochondrial superoxide production.
- CK and Rd increased the production of IL-1β and TNF-α in ATP activated macrophages only in LPS primed cells (both J774 macrophages and peritoneal macrophages).
5.6 Discussion

P2X7 is implicated in signalling between macrophages and other cells of the immune system (521). Upon association with its endogenous ligand, ATP, there is an increase in P2X7 channel activity, which allows for ion flux through this channel. The Ca$^{2+}$ influx, which follows P2X7 activation, is always coupled to K$^+$ efflux (522). The rapid K$^+$ release is a critical trigger for initiation of a number of pro-inflammatory pathways that are activated inside immune cells following stimulation by the danger signal eATP (170). The mitochondrial membrane potential is disrupted due to the rapid decrease in K$^+$ and this leads to increases in the production of ROS (523). Increased ROS production is a well-recognized event following P2X7 activation (524). This is followed by release of cytochrome c from the mitochondrial membrane complex. The release of cytochrome c activates the caspase cascade (including caspase 9, 3 and 7) for executing cell death (525). The optimum activation of all these signals is of prime importance for stimulating and resolving inflammation. An inability to initiate these signals can result in the incomplete clearance of infection leading to a state of chronic inflammation (526). The consequences of modulation of P2X7-related ion flux can affect all the associated pathways, causing an overall change in the ability to resolve inflammation. In the previous chapter, it was described that selected ginsenosides interact with P2X7 after it has been activated by ATP, by binding to a site other than the ATP binding site (allosteric modulation) and can cause an increase in intracellular Ca$^{2+}$ levels. These experiments were performing using P2X7 over-expressed in HEK-293 cells (chapter 4). The recombinant system was ideal for initial screening purposes as it provided an opportunity for the receptor to be studied in isolation from other interacting proteins since HEK-293 cells lack the inflammatory machinery mandatory for eliciting immune responses following the activation of P2X7. For understanding the effect of P2X7 on downstream signalling, these events had to be studied in macrophages; the cells furnished with the appropriate machinery for a complete inflammatory response. Therefore, in
the study described in this chapter, the events following the potentiation of receptor activity were studied in detail using the J774 macrophage cell line as well as rat primary peritoneal macrophages.

The selected ginsenosides were tested for their effect on intracellular Ca\(^{2+}\) concentrations following ATP-induced activation of P2X7. CK and Rd (10 µM each) caused a substantial increase in Ca\(^{2+}\) influx in macrophages while Rb1 and Rh2 did not show any effect on P2X7 mediated Ca\(^{2+}\) influx (Figure 5.5).

LPS is the paradigmatic stimulator of inflammation (527). The presence of LPS-binding domains in the C-terminal domain has been suggested to be responsible for the ability of P2X7 to influence secretion of different immunomodulatory molecules (IL-1β, TNF-α, NO) in LPS-stimulated macrophages (528). Denlinger et al. demonstrated the ability of certain P2X7 derived peptides to bind to LPS in vitro (529). Furthermore, this binding of LPS to P2X7 was shown to inhibit activation of ERK1, ERK2 (extracellular signal-regulated kinases) by LPS in RAW 264.7 macrophages (528). On its own, LPS is known to cause a slow activation of caspase-1, which causes an accumulation of the inactive form of IL-1β. The decrease in intracellular K\(^+\) concentration following activation of P2X7 provides the additional trigger (signal 2) for the complete activation of caspase-1. Thus, P2X7-LPS axis may be responsible for the comprehensive inflammatory response in immune cells such as macrophages.

Therefore, it was expected that treatment of J774 macrophages with LPS would cause an increase in the extent of potentiation provided by CK and Rd. Instead, it was observed that there was no increase in the Ca\(^{2+}\) influx for macrophages exposed to LPS for 4 hours before ATP activation in comparison to non-LPS primed cells. This may suggest that the LPS priming did not affect the P2X7 channel activity directly in J774 macrophages. The channel activity is increased by ginsenosides but there is no additional influx of ions through the channel for macrophages stimulated with LPS. Since LPS treatment did not cause any change in expression
of P2X7 on the surface of cells, the above observations cannot be attributed to the difference in number of P2X7 receptors on the cell surface following the priming event of macrophages.

Involvement of P2X7 in cell death events has been asserted in many studies (530-534). It has been suggested that the transient opening of P2X7 channel by short exposure times to ATP leads to Ca\(^{2+}\) influx (535), Phospholipase D activation (536), ATP-induced ATP release (537), cell fusion and proliferation whereas a prolonged exposure (30 minutes) causes greater Ca\(^{2+}\) influx, activation of transcription factors, surface molecule shedding, intracellular parasite killing and cell death (538). In the present study, macrophages were exposed to ATP for 24 hours in the presence and absence of ginsenosides. The cell viability data from the experiments suggested that CK enhanced the ability of P2X7 to cause cell death at an otherwise non-lethal dose of ATP-concentration. It was reported by Mackenzie et al. in 2005 that brief activation of HEK-P2X7 cells (~ 2 minutes) resulted in “pseudoapoptosis” (450). This term was used because the events following the exposure of these cells to ATP for a short period of time were similar to apoptotic events (increases in cytosolic and mitochondrial calcium, membrane blebbing, actin filament disruption, phosphatidylserine exposure, and disruption of ΔΨ\(_m\)). However, all of these responses were found to be reversible. It was suggested that prolonged exposure of HEK-P2X7 cells to ATP was required for cell death to occur (the mechanism of which was proposed to be calcium independent by the authors) (450). The results in the present study are similar in that J774 macrophages exposed to ATP (500 µM) exhibited Ca\(^{2+}\) influx, increases in ROS and caspase 3/7 activity but did not result in cell death after 24 hours. In the presence of CK, the initial trigger was suitably reinforced so that the cells committed to the cell death pathway.

The ability of Rd to increase P2X7-dependent cell death was evident only in the presence of LPS. It is known that LPS is a very strong inflammatory stimulus but by itself does not cause a complete inflammatory response (in terms of cytokine secretion). In the presence of eATP
though, P2X7 mediated K\(^+\) efflux causes the essential trigger to initiate the inflammatory pathways. It has been shown that LPS stimulated macrophages from P2X7 knockout mice are resistant to ATP-induced cell death and cytokine release (103, 104). Therefore, it has been suggested that the P2X7/LPS axis is responsible for initiation and progression of complete inflammatory/ cell death pathway in macrophages. From the results obtained from the cell viability studies with the selected ginsenosides, CK was consistent in its behaviour in the presence as well as absence of LPS. Rd on the other hand did not have any significant effect on cell viability in non-primed cells. In LPS primed cells Rd was able to cause cell death in 50 % of the J774 macrophages. This may be explained by keeping in mind that P2X7 activation causes K\(^+\) efflux and LPS leads to NLRP3 inflammasome activation. Rd increased Ca\(^{2+}\) influx via ATP activated P2X7. Therefore, it also enhances the K\(^+\) efflux. The increase in K\(^+\) efflux by Rd along with NLRP3 inflammasome activation by LPS together could be a possible tipping point for Rd to express its cell death increasing potential. Therefore, it could be suggested that exposure of macrophages to LPS encourages them to commit to cell death and this event is accentuated by the presence of the ginsenoside Rd. Other ginsenosides tested (Rb1 and Rh2) did not have any significant effect on cell viability of ATP stimulated J774 macrophages with or without LPS priming. From the Ca\(^{2+}\) influx data obtained, this was the expected behaviour of these compounds. It may be suggested that LPS acts downstream of the channel opening and lowers the concentration of ATP required for P2X7 activation. Once the ATP threshold is lowered then Rd opens the channels, causes more Ca\(^{2+}\) influx, and affects cell viability in a manner similar to CK.

In a recent study, LPS treatment of THP-1 macrophages (± ATP) was found to initiate “pyroptosis” within the first 2 hours of LPS exposure (539). Pyroptosis is a form of cell death which involves the maturation and release of IL-1β and IL-18 via activation of caspase-1 (540). Since a number of different events can happen following ATP activation of P2X7 (depending
on the time of exposure, the concentration of ATP), further investigations with varying ATP and Rd concentrations for different time spans are required.

The measurement of Ca\(^{2+}\) influx in this study was carried out over a period of 5 minutes, where cells were exposed to ATP for 240 seconds (Figure 5.1). The MTS assay for measurement of cell death was carried out for 24 hours (Figure 5.8). The cell death experiments described in Chapter 5 show that at 500 µM ATP, the cells do show an increase in the initial Ca\(^{2+}\) influx as well as other apoptotic parameters such as ROS release as well as cytokine production but are unable to commit to cell death even after a 24 hours exposure. In the presence of CK, the initial Ca\(^{2+}\) influx is increased many-fold and this increased Ca\(^{2+}\) concentration seems to increase the magnitude of all the related signals.

An increase in intracellular Ca\(^{2+}\) is known to stimulate opening of the mitochondrial permeability transition pore complex (PTPC) and the activation of caspases (541). The activated caspases can degrade the oxidative phosphorylation complexes in the inner mitochondrial membrane leading to disruption of mitochondrial membrane potential (\(\Delta \Psi_m\)) (542). An increase in ROS generally accompanies the progressive loss of \(\Delta \Psi_m\) (543). The increase in cellular ROS and mitochondrial superoxide production by ginsenoside CK in this study confirmed that the potentiation of P2X7 caused the mitochondrial Ca\(^{2+}\) overload (Figure 5.10 and Figure 5.13). This further led to increased caspase 3/7 activity (CK + ATP results in 100 % cells to be caspase 3/7 active after 6 hours) (Figure 5.17). Thus, in the presence of ATP concentrations where macrophages activate their stress response but the cells fail to die, the presence of CK would provide the required additional trigger for them to commit to the cell death pathway.

In the presence of LPS, another pathway is switched on in parallel. The priming of macrophages with LPS alone cannot initiate caspase-1 activation (544). For caspase-1 to be active, an increase in K\(^+\) efflux and ROS production caused by ATP-induced P2X7 activation
is known to act as the required second signal or hit (545). An increase in this second signal would presumably increase caspase-1 activation as well.

In a recent study, it has been suggested that there is cross-talk between the apoptotic pathway and pyroptotic pathways (546). If a cell is being challenged by both apoptotic (ATP) and pyroptotic (LPS) stimuli, this could result in activation of caspase 3/7 (via ATP) and caspase-1 (via LPS). It has been suggested that in this case, the pyroptotic pathway would be inhibited and caspase-1 would activate caspase 3/7. Therefore, the pro-inflammatory signals would be switched off and the cells involved would undergo apoptosis. Therefore, the results described in Chapter 5 may be understood in light of the observations made by Taabazuing et al. (546). CK and Rd increase K+ efflux (within few seconds) and ROS production (15-105 minutes) in ATP-activated macrophages. In the presence of LPS (4 hours priming), these signals activate caspase-1 (not directly measured) and cause increased production of pro-inflammatory cytokines. The caspase 3/7 enzymes are maximally active within 6 hours. Thus the 24 hours’ time frame of the experiment is enough for CK to cause macrophage death even in the absence of LPS. In the LPS primed cells though, the caspase-1 which was active within the 4 hours 30 minutes time frame (when the cytokines were measured) would now switch off the pyrogenic pathway and instead activate the caspase 3/7 enzymes further. This could explain the behaviour of ginsenoside Rd which in the absence of LPS priming is unable to help the cells cross the “point-of-no-return” but in LPS primed cells, seems to mimic the ability of CK to promote cell death. This could be because the activate caspase-1 from the pyrototic pathway is providing the additional push over the tipping point required for Rd treated macrophages to die.

5.7 Conclusion

From the present study, it could be concluded that the major ginseng metabolite CK potentiated P2X7 responses in macrophages. The potentiation of P2X7 by CK increased the Ca2+ influx and ROS production, thereby activating the caspase 3/7 in these cells. Activation of caspase
3/7 by CK then led to macrophage death in the presence of an otherwise non-lethal dose of ATP (500 µM). These results indicated that CK encouraged the cells to commit to cell death pathway under stress conditions. In the presence of the pro-inflammatory stimulus, LPS, Rd also enables cell to undergo death, in a manner similar to CK. Presence of pro-pyroptotic signal (LPS) along with apoptotic stimulus (eATP) was observed to increase the pro-inflammatory cytokines secretion and this cytokine release was further enhanced by ginsenosides CK as well as Rd. The coalition of these enhanced apoptotic and pyroptotic signals in the presence of ginsenosides may provide a strong enough trigger for the macrophages to die. These results indicate that the bioactive components of Panax ginseng can modulate P2X7 activity, thus providing a scientific explanation to the anti-viral, anti-cancer and anti-inflammatory properties of this medicinal herb.
CHAPTER 6
GENERAL DISCUSSION

The homomeric P2X7 receptor belongs to P2X receptor family of ligand gated ion channels sensitive to relatively high concentrations of extracellular ATP (> 100 µM) (547). P2X7 has received considerable attention as a potential drug target because of its involvement in the pathogenesis of inflammatory disorders as a critical mediator of the inflammasome complex (185). This has led to the identification and characterisation of a number of novel, structurally diverse modulators (233). Pharmacological profiles of various characterised modulators are well defined and the IC$_{50}$/EC$_{50}$ values for these compounds vary depending upon the chemical structure and the mammalian species it is tested in. For instance, A740003 has an IC$_{50}$ of 0.040 µM for hP2X7 while it is 0.020 µM for rat P2X7 (203). Brilliant Blue G non-competitively inhibits human and rat P2X7 with IC$_{50}$ values of 0.2 and 0.001 µM respectively (548). Similarly, P2X7 agonists ATP and BzATP are 10 times and 100 times more potent in rats than in mice (549). In spite of a nanomolar/micromolar potency of some of the P2X7 antagonists tested, there is a lack of specificity for many as these can still affect other purinergic receptors (550). There is also a great variation in the agonist potency among various species which further impedes studies relating this receptor (551).

Modulating P2X7 using pharmacological and genetic approaches alters inflammatory cytokine secretion and cell death in immune cells (552-556). For instance, inhibition of P2X7 by A438079 reduced the pro-inflammatory effects of heat shock protein (HSP 90) in subarachnoid haemorrhage (557). Inhibition of P2X7 by LL-37 (an anti-microbial peptide) decreased the production of pro-inflammatory cytokines (558). P2X selective antagonists NF449 and A438079 inhibited HIV-1 mediated infection and production of inflammatory cytokines IL-10 and IL-1β in a human tonsil explant model (559). The P2X7 antagonist A8044598 reduced
inflammation in brain and liver of C57BL/6J mice exposed to chronic ethanol and high fat diet (560). Blockade of P2X7 by A438079 was also found to inhibit cytokine induced colitis of human colonic mucosa (553). P2X7 receptors have been reported to trigger phagocytosis and regulate cell death (561). These studies indicate that modulation of P2X7 related signalling pathways can have important therapeutic implications in pathological conditions leading to inflammation and cell death. Therefore, identification and characterisation of novel modulators that target P2X7 specifically would be beneficial.

In this thesis, an attempt was made to describe the ability of two completely distinct sets of compounds, tetracycline derivatives (MINO and DOX) and ginseng derived compounds (CK and Rd) to modulate P2X7 receptor activity. Both these sets of compounds were previously reported to have a role in the regulation of inflammatory processes, but this study provided evidence of a possible common molecular target; the P2X7 receptor.

The lipophilic microglial inhibitor minocycline (MINO) is known to inhibit the activation and proliferation of microglia, thereby down regulating neuroinflammation and neuropathic pain (562-565). Microglia are believed to exist in two polar states: the immunomodulatory (M1) state and pro-inflammatory state (M2). It is suggested that MINO selectively inhibits the microglia from converting to the M2 state (566). Several attempts have been made to identify and characterise molecular targets of MINO but no protein has so far been established as an exclusive target for MINO action (301, 337, 352, 567). It has been suggested that microglia derived Fas-ligand mediates immunoreactive responses and recruits’ microglia to the sites of lesions via P2X7 (568). MINO was found to downregulate the Fas/P2X7 mediated microembolic injury during ischaemia (569). It was observed that P2X7 activation led to the accumulation and subsequent release of Fas ligand in conditions of cerebral ischemia (570). In this study, pharmacological evaluation of P2X7 as a possible direct target for MINO was not considered (571). In another recent study, the MINO-mediated reduction in microglia
activation was reported to be accompanied by reduction in levels of TLR-2, its adaptor protein MyD88, as well as the NLRP3 components of the inflammasome (572). The P2X7/NLRP3 axis is central to inflammatory signalling. The downregulation of NLRP3 protein levels by MINO may suggest an action of MINO upstream of the inflammasome assembly.

Chapter 3 described the pharmacological characterisation of MINO in order to evaluate the possibility of P2X7 being directly affected by this drug. The results of this study indicated that MINO downregulated P2X7 channel activity (Ca\(^{2+}\) influx, Figure 3.2) and membrane pore formation (YOPRO-1 uptake, Figure 3.6). The P2X7 associated physiological responses, including cytokine secretion and cell death, were inhibited by MINO. A detailed pharmacological evaluation of MINO indicated that this tetracycline derivative inhibited the efficacy of the agonist ATP towards P2X7 receptor at relatively high concentrations with an IC\(_{50}\) ~ 32.6 µM. It inhibited P2X7 and P2X4 in non-competitive allosteric manner. It was not exclusively selective for P2X7 as closely related P2X4 and P2Y responses were also blocked by the drug. It was suggested inhibition of P2X7 may prevent loss of astroglia by enhancing PARP1 activity in the molecular layer of the dentate gyrus following status epilepticus.

Another study conducted by Alano et al. in 2006 found that MINO inhibits poly (ADP-ribose) polymerase-1 (PARP-1) at nanomolar concentrations. This study reported that micromolar or sub-micromolar concentrations did not affect PARP-1 activity (300). The results in this thesis (Chapter 3) describe that MINO downregulates P2X7 function at high micromolar concentrations. At concentrations below 25µM (results not shown), MINO loses its inhibitory properties towards P2X receptors. Considering all these studies, it could be reasoned that MINO at higher concentrations targets purinergic receptors, which can then inhibit anti-apoptotic PARP-1 indirectly and down regulate pro-apoptotic TLR-1 and NLRP-3 proteins, preserving the M1 phase of microglia. On the other hand, at low concentration, MINO inhibits PARP-1 and increase pro-inflammatory state (M2). So, even though MINO may not be
recognized as a selective, specific antagonist of P2X7, it can be inferred that P2X7 is one of the targets of this multi-target drug.

Conventionally, drugs were intended to target a single cellular component/protein ("on-target" approach), with the purpose of achieving high selectivity for that distinct protein and reducing the chances of any side effects (off-target effects) (573). Due to this consideration, compounds interacting with multiple targets were often discouraged and labelled as “dirty drugs” (574). However, the intricacies of the existing incurable diseases such as neurodegenerative disorders, cancers and mental health problems have clearly proved that such single-target drugs may not be sufficient for therapeutic purposes (575). On the other hand, studies have shown that multi-targets drugs may in fact have a safer profile in these complex pathologies (576, 577). Between the years 2015-2017, 21 % of the total new molecular entities approved by the US Food and Drug Administration (FDA) were multi-target drugs (574). Therefore, microglial inhibitor MINO could be targeting an array of yet undiscovered proteins, which are acting at undefined positions within the P2X7 inflammatory cascade.

An insight into the structure-function relationship was also provided by the interaction of structural analogues of tetracycline; closely related MINO and DOX as well as the glycylcycline TIGE with P2X7. Tetracycline and its analogues share a basic chemical structure made up of tetracyclic naphthacene carboxamide ring system (578). The presence of dimethylamine group at C4 in the ring A is said to be essential for the antimicrobial activity of tetracycline (579). The ROS-scavenging abilities of these compounds are generally attributed to the phenol rings which react with free radicals to generate a resonance stabilised, unreactive phenolic radical (580). No definite structural attribute has been assigned to the anti-inflammatory properties of these compounds at this time. In this thesis, the tetracycline derivatives showing P2X7 inhibitory responses were MINO and DOX. The structural difference between these two compounds and tetracycline is the modification of C-7 of ring D
in MINO and C-6 in ring C in DOX. The methyl group on the upper peripheral zone of these compounds could be responsible for binding to P2X7 receptor. Addition of the t-butyrglycylamido group on position C-9 in TIGE is assumed to contribute to its better antibiotic activity, but in this study, TIGE did not inhibit P2X7 activity, indicating that this structural variation may not contribute to anti-inflammatory properties of tetracyclines. Docking studies are essential for providing further insight into the structure-activity relationship of these compounds so that structural analogues with better specificity, strength and selectivity could be generated to target P2X7 mediated inflammatory action.

Inflammation is a protective response elicited by the body in order to defend the living system against any incoming disturbance or potential danger. The cells of the immune system collaborate in a precisely coordinated manner to fight infection as well as resolve and repair any damage caused by the inflammatory response. Ideally, inflammatory responses are perfectly balanced, which means that the inflammatory cells and other components involved in immune system-pathogen combat are cleared immediately after the infection is resolved. In case of sub-optimal inflammatory responses, resolution of the inflammatory cells is not very efficient, and the neutrophils and macrophages recruited for eliminating the invading pathogen persist long after the actual infecting agent has been removed. This imperfect resolution of inflammation leads to chronic inflammation. Inhibiting P2X7 responses may be beneficial in suppressing the initial acute inflammatory responses but in case of chronic inflammation, an up regulation of P2X7 could help in resolving inflammation, thereby helping to ameliorate or abolish the symptoms related to chronic inflammation. The second part of this thesis (Chapters 4 and 5) describes novel positive modulators of P2X receptors.

Over activation of P2X7 has been reported to have positive effects under certain conditions. For instance, the ATP-P2X7 pathway activates the p38/JNK/ATF-2 signalling pathway; thereby protecting bone marrow derived macrophages (BMDMs) and the macrophage cell line
RAW 264.7 from vascular stomatitis virus (VSV) mediated cell death via decreasing viral replication in vitro. Similar results were obtained in vivo where a decrease in viral replication was observed on activation of P2X7 in wild type mice (581). That viral suppression is P2X7 mediated is confirmed by the absence of any anti-viral activity in P2X7 knockout mice (581). Thus, potentiation of P2X7 could have anti-viral effects. P2X7 activation has been shown to have anti-bacterial effects as well. A decrease in bacterial load in epithelial cells and macrophages is observed on activation of P2X7 by extracellular ATP following infection by various Chlamydieae strains (233). P2X7 knockout mice have been found to be more susceptible to Chlamydiae infection in comparison to their wild type counterparts (582). Similarly, P2X7 has been linked with Mycobacterial infections. Loss-of-function mutation of P2X7 is associated with increased susceptibility to tuberculosis infection (583). It is suggested that the loss of functional P2X7 receptors impairs the capacity of macrophages to eliminate intracellular bacteria (584). It has been shown that ATP activation of P2X7 causes bacterial clearance in infected macrophages by increasing activation of phospholipase D and apoptosis of infected cells. Genetic deletion of P2X7 was observed to decrease the susceptibility of infection by H37Rv strain of Mycobacterium tuberculi (585).

P2X7 receptor is also known to play both anti-tumour and pro-tumour roles depending upon the concentration of ATP in the interstitium of tumours, amount of ectonucleotidases in the immune cells, tumours and stromal cells as well as the type of purinergic receptors expressed by the tumour cells (424). Therefore, the role of P2X7 in cancer progression is often debated. Overstimulation of P2X7 resulting in tumour cell death has been demonstrated in a number of preclinical studies. (202, 586). In clinical trials though, increasing ATP in the extracellular matrix has not been shown to have many beneficial effects. It is suggested that tumour cells may be resistant to extracellular ATP because of disengagement of P2X7 from intracellular cell death pathways in some cancers. Therefore, potentiation of P2X7 could be a useful
approach in combating cancer in scenarios where the receptors are less responsive to extracellular ATP.

Mello et al. in 2017 demonstrated that potentiation of P2X7 responses by hyperthermia changes plasma membrane fluidity resulting in an increased membrane pore and dilation following ATP-P2X7 activation. In combination with chemotherapeutic agents such as cisplatin and mitomycin C, potentiation of P2X7 via hyperthermia was seen to enhance the efficacy of these antitumor drugs (587). Potentiation of P2X7 responses by selected ginsenosides characterised as pharmacological modulators in this thesis (Chapter 4 and Chapter 5) may also function in a manner similar to hyperthermia and could be used in combination with other anti-tumour agents.

The results from chapter 4 indicated that selected ginsenosides increased the P2X7 channel activity and related signalling in HEK-293 (transfected) and macrophages. Not only do these results provide an explanation for some of the immunomodulatory properties of Panax ginseng, they also identified two naturally occurring chemical compounds that can decrease the EC50 of ATP for P2X7 activation, thereby enhancing all downstream events controlled by this receptor. Of the 4 ginsenosides tested, CK and Rd consistently caused pronounced enhancement of PX7 related effects. The similar efficacy of these two ginsenosides might suggest a common binding site for both ginsenosides on P2X7. A comparison of the structure of the four ginsenosides tested and the varying magnitude of their effects on P2X7 can shed some light on the structure-function relationship of these drugs with the receptor. For instance, positional isomers CK and Rh2 have similar structures except for the position of one glucose molecule. This subtle change in structure probably affects the orientation in which the two compounds lodge themselves in the binding site on P2X7 and this differential binding defines the levels of P2X7 enhancement elicited by the two compounds. Thus, the in-depth investigation of selected ginsenosides in
chapter 4 brought forward two new P2X7 modulators and their pharmacological properties which could be beneficial in future drug development.

In chapter 5, the physiological relevance of the potentiation of P2X7 by ginsenosides CK and Rd was validated. In the presence of sub-lethal doses of ATP, the apoptotic machinery was switched on, but the trigger was not large enough for the cell to die. In the presence of pyroptotic stimuli (LPS), the pro-inflammatory pathway was activated but cell death did not occur. In the presence of CK (ATP ± LPS), the pro-apoptotic signals were high enough to help the cells to reach a point-of-no-return and thus die. In light of the recent study suggesting a cross-talk between pyroptosis and apoptosis (where it has been suggested that caspase-1 stimulates caspase 3/7) (546), it could be suggested that large activation of caspase 3/7 at an early time point in the presence of CK might help in preventing the pro-inflammatory pathway and re-routing the cells towards apoptosis. Taken together, the results from Chapter 4 and Chapter 5 have added to our knowledge of the pharmacological properties and physiological relevance of bioactive components of ginseng, thereby providing a scientific validation to some of the reported immunomodulatory properties of ginseng. P2X7 activation is known to have beneficial anti-viral, anti-fungal, anti-bacterial as well as anti-cancer effects. Generation of structural analogues of CK and Rd with greater specificity for P2X7 can be considered in order to generate better therapeutic tools to target P2X7.

Pacheco et al. have shown that the rate of entry of the photosensitizer methylene blue (MB) in macrophages increases from 4.2 % to 90.2 % following ATP activation of P2X7 membrane pores (588). The authors of this study have suggested that the pore associated with P2X7R could as a drug delivery system to increase the passage of hydrophilic drugs into cells expressing P2X7. Thus, hydrophilic drugs (with molecular mass less than 900 Da) can be delivered into specific cells by opening up the P2X7 associated membrane pore. Increasing the
dilation of this pore further by positive modulators such as CK and Rd may additionally enhance the efficacy of this drug delivery system.

General Conclusions

In conclusion, the findings from the present study have provided evidence in support of P2X7 as a molecular target for MINO as well as ginseng compounds CK and Rd, even though these compounds have contrasting modulatory effects on P2X7 activity. The results from these studies have added to the pharmacological knowledge related to P2X7 and P2X4 receptors. This knowledge can prove beneficial in drug development. In the physiological context, a healthy state can be maintained if inflammatory responses are optimum. The inflammatory responses should be strong enough to deal with the external challenge but should be controlled so as self-cells are not harmed. Therefore, inhibition of overactive P2X7 by the negative modulator MINO may have anti-inflammatory effects. On the other hand, potentiation of P2X7 by CK and Rd may be beneficial in boosting immunity in the case of viral, microbial, parasitic, fungal infections as well in some forms of cancer. This thesis has provided an insight into the pharmacological properties of novel P2X7 modulators and the information from the studies described herein may prove useful in generating more specific therapeutic tools to target the multifaceted inflammatory P2X7 receptor.

Future Directions

The direct effect of MINO on P2X channels has not been established. It is proposed that MINO might affect the common pore formation pathway mediated by P2X receptors but the confirmation of the effect of MINO on P2X7 using electrophysiology would be useful in order to exactly describe the mode of action of this drug. In Chapter 4, the direct effect of ginsenosides on the P2X4 channel has been established using an automated patch clamp system (IonFlux). The selectivity of selected ginsenosides for P2X7 was evaluated by testing them
P2X4, P2X2 and P2Y. It would be useful to test these drugs on other P2X receptors as well (P2X1, P2X3, P2X5 and P2X6). This could provide a better understanding of the structure-function relationship and binding properties of ginsenosides for purinergic receptors. Computational docking studies would be useful for predicting the energetically and geometrically feasible binding sites for ginsenosides on these receptors. In Chapter 5, CK and Rd have been shown to help the macrophages challenged by stress to commit to a cell death response. The entire cell death pathway has not been completely characterised in this work. A direct measure of apoptosis using assays such as TUNEL to confirm nicking of DNA would provide confirmation about the type of cell death induced by these receptors. Also, in the pro-inflammatory pathway, further testing for any direct effects of ginsenosides on caspase-1 activity would be useful. The data in the present study was not validated on human cells (macrophages or monocytes). The initial screening was done using HEK-293 cells over-expressing the receptors of interest. The studies were further validated using immortalised cell lines (BV2 microglial and J774 macrophage cell lines). Apoptotic pathways are often altered in immortalised cell lines, therefore validating the results in primary cells or in vivo would be a good approach. The primary macrophages from rat were employed to confirm some of the results obtained with cell lines (Chapter 3 and 5). Macrophages and microglia express both P2X7 and P2X4. In order to determine the effect of drugs on one receptor type independently of the other, CRISPR/Cas9 gene editing system can be used. The CRISPR/Cas9 can silence one purinergic receptor type (example P2X7) so that the selectivity of the receptor responses (example P2X4) can be studied independently of the other. In Chapter 4, CRISPR/cas9 can be used to silence the P2X7 receptors in J774 macrophages or BV2 microglia to measure the effect of ginsenosides on P2X4 receptors in these cells. Similarly, in Chapter 3 and 5, the P2X4 receptors can be silenced in J774 macrophage cells to test the ginsenosides on P2X7 mediated signalling pathways. It is also known that drug interactions can be different depending on cells.
studied and species studied. Therefore, the best possible approach would be to test these drugs in immune cells of human origin (human macrophages or monocytes).
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APPENDICES

Cell Line Information

A. HEK-293 cells

Cell line name: HEK-293

Description:

- Species: Human, *Homo sapiens*
- Tissue: Embryonic kidney
- Age: foetus

Depositor: ATCC (procured by Dr Leanne Stokes)

Biosafety level: 2

Growth mode: Adherent

Morphology: Epithelial-like

Propagation: Complete DMEM: F12 medium: DMEM:F12 + 10 % FBS + 1 % Pen Strep (+5,000 Units/ml Pen; +5,000 µg/ml Strep)

Sub culturing:

- Ratio: Split 1:8 to 1:10
- Seeding: at 2–4⇥10, 000 cells/cm². Remove spent medium, rinse with PBS (w/o Ca and Mg), add fresh 0.05 % trypsin/EDTA, incubate at 37°C until the cells detach. Add fresh medium, aspirate, and transfer into new flask. Incubate in incubator at 37°C (and 5 % (v/v) CO²).
Preservation: FBS 90 %; DMSO 10 % (v/v)

B. J774A.1 macrophage cell line

Cell line name: J774

Description:

- Species: Mus musculus, mouse
- Tissue: ascites
- Disease: reticulum cell sarcoma

Depositor: ATCC (kindly provided by Dr. Ray Helliwell at RMIT)

Biosafety level: 1

Growth mode: Adherent

Morphology: Epithelial-like

Propagation: Complete RPMI medium: RPMI + 10 % FBS + 1 % Pen Strep (+5,000 Units/ml Pen; +5,000 μg/ml Strep)

Subculturing:

- Ratio: Split 1:10
- Seeding: at 2−4×10,000 cells/cm². Remove spent medium, rinse with PBS (w/o Ca and Mg), add fresh 0.05 % trypsin/EDTA, incubate at 37°C until the cells detach. Add fresh medium, aspirate, and transfer into new flask. Incubate in incubator at 37°C (and 5 % (v/v) CO²).
- Preservation: FBS 90 %; DMSO 10 % (v/v)
C. BV-2 microglia cell line

Cell line name: BV-2

Description:

- **Species:** *Mus musculus*, mouse
- **Cell line status:** Transformed (recombinant retrovirus J2)

Biosafety level: 2

**GROWTH MODE:** ADHERENT

**Morphology:**

Propagation: Complete DMEM: F12 medium: DMEM: F12 + 10% FBS + 1% Pen Strep (+5,000 units/ml Pen; +5,000 μg/ml Strep)

**Subculturing:**

- **Ratio:** Split 1:3 to 1:8
- **Seeding:** at 2–4⇥10,000 cells/cm². Remove spent medium, rinse with PBS (w/o Ca and Mg), add fresh 0.05% trypsin/EDTA, incubate at 37°C until the cells detach. Add fresh medium, aspirate, and transfer into new flask. Incubate in incubator at 37°C (and 5% (v/v) CO₂).
- **Preservation:** FBS 90%; DMSO 10% (v/v)
D. Buffers

Phosphate Buffer Saline (PBS, pH 7.3)

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<th>Reagents</th>
<th>Concentration(1X)</th>
<th>Amount to be added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NaCl</td>
<td>137 mM</td>
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<tr>
<td>2.</td>
<td>KCl</td>
<td>2.7 mM</td>
<td>0.2 g</td>
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<tr>
<td>3.</td>
<td>Na$_2$HPO$_4$</td>
<td>10 mM</td>
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<td>4.</td>
<td>KH$_2$PO$_4$</td>
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<td>5.</td>
<td>CaCl$_2$.2H$_2$O</td>
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<td>6.</td>
<td>MgCl$_2$.6H$_2$O</td>
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<td>1.0 g</td>
</tr>
</tbody>
</table>

The above salts were weighed and dissolved in 800 ml distilled water. The pH was adjusted to 7.3 with conc. HCl and then the volume was adjusted to 1 L using distilled water. The buffer was then sterilized by autoclaving at 15 psi for 20 minutes.

Electrolyte Low Divalent Buffer (ELDV, pH 7.3)

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<tr>
<td>2.</td>
<td>HEPES</td>
<td>10 mM</td>
<td>2.38 g</td>
</tr>
<tr>
<td>3.</td>
<td>Glucose</td>
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<td>2.34 g</td>
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<td>4.</td>
<td>KCl</td>
<td>5 mM</td>
<td>5 ml of 1M stock</td>
</tr>
<tr>
<td>5.</td>
<td>CaCl$_2$.2H$_2$O</td>
<td>0.2 mM</td>
<td>200 µL of 1 M stock</td>
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</table>

The above salts were weighed and dissolved in 800 ml distilled water. The pH was adjusted to 7.3 with 4 M NaOH and then the volume was adjusted to 1 L using distilled water.
### Physiological buffer ($E_{\text{total}}$, pH 7.3)

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<td>8.47 g</td>
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<tr>
<td>2.</td>
<td>HEPES</td>
<td>10 mM</td>
<td>2.38 g</td>
</tr>
<tr>
<td>3.</td>
<td>Glucose</td>
<td>13 mM</td>
<td>2.34 g</td>
</tr>
<tr>
<td>4.</td>
<td>KCl</td>
<td>5 mM</td>
<td>5 ml of 1M stock</td>
</tr>
<tr>
<td>5.</td>
<td>CaCl$_2$·2H$_2$O</td>
<td>2 mM</td>
<td>2 ml of 1 M stock</td>
</tr>
<tr>
<td>6.</td>
<td>MgCl$_2$·6H$_2$O</td>
<td>1 mM</td>
<td>1 ml of 1M stock</td>
</tr>
</tbody>
</table>

The above salts were weighed and dissolved in 800 ml distilled water. The pH was adjusted to 7.3 with 4 M NaOH and then the volume was adjusted to 1 L using distilled water.

### Krebs-HEPES buffer (pH 7.3)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Reagents</th>
<th>Concentration(1X)</th>
<th>Amount to be added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NaCl</td>
<td>140 mM</td>
<td>8.18 g</td>
</tr>
<tr>
<td>2.</td>
<td>KCl</td>
<td>2.8 mM</td>
<td>0.208 g</td>
</tr>
<tr>
<td>3.</td>
<td>HEPES</td>
<td>10 mM</td>
<td>2.38 g</td>
</tr>
<tr>
<td>4.</td>
<td>Glucose</td>
<td>10 mM</td>
<td>1.8 g</td>
</tr>
<tr>
<td>5.</td>
<td>CaCl$_2$·2H$_2$O</td>
<td>2 mM</td>
<td>0.294 g</td>
</tr>
<tr>
<td>6.</td>
<td>MgCl$_2$·6H$_2$O</td>
<td>2 mM</td>
<td>0.406 g</td>
</tr>
</tbody>
</table>

The above salts were weighed and dissolved in 800 ml distilled water. The pH was adjusted to 7.3 with 4 M NaOH and then the volume was adjusted to 1 L using distilled water.
HBSS-HEPES Buffer (pH 7.3)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Reagents</th>
<th>Concentration(1X)</th>
<th>Amount to be added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>HEPES</td>
<td>20 mM</td>
<td>1 ml of 1M solution</td>
</tr>
<tr>
<td>2.</td>
<td>CaCl₂·2H₂O</td>
<td>2 mM</td>
<td>100 µL of 1M solution</td>
</tr>
<tr>
<td>3.</td>
<td>MgCl₂·6H₂O</td>
<td>1.2 mM</td>
<td>60 µL of 1M solution</td>
</tr>
</tbody>
</table>

This buffer was prepared fresh (50 ml) for every experiment. The above solutions were added to 45 ml 1 X HBSS (Ca²⁺, Mg²⁺, phenol red free) buffer and the pH was adjusted to 7.3 with 10 M NaOH. The volume was made to 50 ml using HBSS. The buffer was syringe filtered (0.22 µM Millipore filters) before use.

Ammonium chloride lyse (10X concentration)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Reagents</th>
<th>Amount to be added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NH₄Cl</td>
<td>8.02g</td>
</tr>
<tr>
<td>2.</td>
<td>NaHCO₃</td>
<td>0.84g</td>
</tr>
<tr>
<td>3.</td>
<td>EDTA (disodium)</td>
<td>0.37g</td>
</tr>
</tbody>
</table>

Volume was made up to 100ml with Millipore water. Working solution was prepared by diluting 10 X concentrate with 90 ml Millipore water. Solution was refrigerated until use.