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Expression and function of the P2X7 receptor on human malignant cell lines

Safina Gadeock

University of Wollongong

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Expression and function of the P2X7 receptor on human malignant cell lines

A thesis submitted in (partial) fulfilment of the requirements of the degree of Master of Science (Research)

University of Wollongong

Safina Gadeock, MSc Biotechnology, BSc Biotechnology

School of Biological Sciences

Faculty of Science

August 2010
Certification

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the degree of Master of Science (Research). It does not include any material previously published by another person except where due reference is made in the text. The experimental work described in this thesis is original and has not been submitted for a degree to any other University.

Safina Gadeock
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Acknowledgements

I would like to express my deep and sincere gratitude to my supervisor, Dr Ronald Sluyter, for his detailed and constructive comments during the preparation of this thesis, and for his constant encouragement and guidance throughout the year. Sincerest thanks to Aleta Pupovac, Iman Jalilian and Bin Wang for providing ample support with experiments, reviewing drafts and being the most awesome labmates ever. I owe my loving thanks to my family and all my friends, for their encouragement and moral support which kept me going in difficult moments.

Publications arising from this thesis


Presentations during the course of MSc. Research

1. Australian Society of Immunology Annual Conference, Bowral, 2009, Title of Seminar: TGF-β1 abrogates the up-regulation of the P2X7 receptor by IFN-γ and LPS in leukemic THP-1 monocytes.

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## Abbreviations

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<tr>
<td>2MeSATP</td>
<td>2-methylthioadenosine 5’-triphosphate</td>
</tr>
<tr>
<td>7AAD</td>
<td>7-amino-actinomycin-D</td>
</tr>
<tr>
<td>ÜmeATP</td>
<td>Ü-ethyl adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a CARD</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5’-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>ATP-γ-S</td>
<td>Adenosine 5’O-(3-thio) triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BzATP</td>
<td>2(3’O-(4-benzoylbenzoyl) adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ECCC</td>
<td>European Collection of Cell Cultures</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>h</td>
<td>Hour (s)</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interlukin</td>
</tr>
<tr>
<td>iPLA₂</td>
<td>Ion phospholipase A₂</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>Isatin</td>
<td>5,7-dibromo-N-(p-hydroxymethyl benzyl) isatin</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>KN-62</td>
<td>1-(N,O-bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl)-4-phenyl piperazine</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Min</td>
<td>Minute (s)</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenytetrazolium bromide</td>
</tr>
<tr>
<td>NALP</td>
<td>Nucleotide-binding domain and leucine-rich repeat containing a pyrin domain</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa beta</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>RONS</td>
<td>Reactive oxygen and nitrogen reactive species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-Polymerase chain reaction</td>
</tr>
<tr>
<td>s</td>
<td>Second (s)</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>----------------------------</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine 5′diphosphate</td>
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<tr>
<td>UTP</td>
<td>Uridine 5′triphosphate</td>
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Abstract

The P2X7 receptor is a ligand-gated ion channel present on normal and malignant cells of hematopoietic and epithelial origins. Activation of the P2X7 receptor by extracellular adenosine 5'-triphosphate (ATP) causes the rapid flux of Na⁺, K⁺ and Ca²⁺. Continued activation of P2X7 allows the formation of large, non-specific pores, allowing the uptake of organic molecules including cation dyes. In addition, activation of P2X7 leads to a number of downstream signalling events which includes the maturation and release of interleukin (IL)-1β, as well as cell proliferation and death. As a result of these latter roles of P2X7 in cell survival, this receptor is attracting considerable interest for its growth-promoting and growth-inhibitory roles in cancer. Therefore, the aim of this project was to study the expression and function of the P2X7 receptor in myeloid leukemic and epithelial malignant cancer cell lines.

Previous work in our laboratory demonstrated that the immunomodulatory cytokine, transforming growth factor (TGF)-β1 prevents the up-regulation of P2X7 in the human myeloid leukemic cell line, THP-1 by interferon (IFN)-γ and lipopolysaccharide (LPS). The mechanism of action of TGF-β1 in this process however was never elucidated. Therefore, the first part of my study aimed to determine the mechanism of action of TGF-β1 on the up-regulation of P2X7 expression and function in THP-1 cells differentiated with IFN-γ and LPS. Cell-surface molecules including P2X7 were examined by immunofluorescence staining. Total P2X7 protein and mRNA was assessed by immunoblotting and RT-PCR respectively. P2X7 function was evaluated by ATP-induced cation dye uptake measurements. IL-1β release was measured by ELISA. Cell-surface P2X7 was present on THP-1 cells differentiated for 3 days with IFN-γ and LPS but not on undifferentiated THP-1 cells. Similarly, ATP
induced ethidium$^+$ uptake into differentiated but not undifferentiated THP-1 cells. Co-incubation of cells with TGF-β1 plus IFN-γ and LPS prevented the up-regulation of P2X7 expression and ATP-induced ethidium$^+$ uptake. Moreover, ATP-induced YO-PRO-1$^{2+}$ uptake and IL-1β release were abrogated in IFN-γ and LPS-treated cells co-incubated with TGF-β1. Of note, TGF-β1 abrogated the amount of total P2X7 protein and mRNA induced by IFN-γ and LPS. Finally, TGF-β1 prevented the up-regulation of cell-surface CD86, but not other differentiation markers (CD14 and MHC class II), by IFN-γ and LPS. Collectively, these results indicate that TGF-β1 prevents the up-regulation of P2X7 by IFN-γ and LPS in THP-1 monocytes by preventing P2X7 transcription and subsequent translation. Moreover, this effect of TGF-β1 is not due to general impairment of THP-1 cell differentiation by IFN-γ and LPS. This suggests that TGF-β1 may limit P2X7-mediated processes during inflammation and immunity, as well as in cancer cells.

Previous work by others has indicated that ATP and 2(3$\beta$-O-(4-benzoylbenzoyl)adenosine 5$\beta$-triphosphate (BzATP) induces the death of the human epithelial colon carcinoma cell lines, HCT-8 and Caco-2 in a manner characteristic of P2X7 activation. However a direct role for this receptor in this process was not established. Therefore, the second part of my study aimed to determine if HCT-8 and Caco-2 cells expressed functional P2X7. Cell-surface and total P2X7 was examined by immunofluorescence staining and immunoblotting respectively. P2X7 function was evaluated by ATP- and BzATP-induced ethidium$^+$ uptake measurements. Reduction in cell numbers, as an indirect measure of cell death, was evaluated using a tetrazolium-based colorimetric assay. The human multiple myeloma cell line, RPMI 8226, was used as a positive control. HCT-8 and Caco-2 cells expressed low levels of cell-surface P2X7. Whole
lysates of these cells expressed low levels of the full length (75 kDa) P2X7, but higher levels of a 42 kDa P2X7 variant. In contrast, RPMI 8226 cells expressed relatively high levels of cell-surface P2X7, as well as full-length P2X7. ATP and BzATP consistently failed to induce ethidium$^+$ uptake in HCT-8 and Caco-2 despite extended incubation times of up to 30 min, and the use of several media known to potentiate P2X7 activation. ATP and BzATP also failed to cause significant death in both the cell lines. In contrast, ATP and BzATP induced ethidium$^+$ uptake and death in RPMI 8226 cells. IFN-$\gamma$ failed to upregulate P2X7 expression and function in HCT-8 cells. Collectively, these results indicate the absence of functional P2X7 in HCT-8 and Caco-2 cells despite the presence of low levels of cell-surface P2X7. The high proportion of a 42 kDa P2X7 variant provides a possible explanation for the lack of functional P2X7 in these cells.

The presence of functional P2X7 in primary myeloid leukemias has been reported by others; however cell line models of myeloid leukemia that constitutively expresses functional P2X7 are limited. Therefore, the final part of my study aimed to determine if the human myeloid leukemic cell line KG-1 expresses functional P2X7. Cell-surface P2X7 was examined by immunofluorescence staining. Total P2X7 protein and mRNA was assessed by immunoblotting and RT-PCR respectively. P2X7 function was evaluated by ATP-induced ethidium$^+$ uptake measurements. Reduction in cell numbers was evaluated using a tetrazolium-based colorimetric assay and cell death was confirmed using a trypan blue exclusion assay and morphological analysis. KG-1 cells expressed low levels of cell-surface P2X7, as well as low levels of P2X7 protein and mRNA. Both, ATP and BzATP consistently induced ethidium$^+$ uptake in KCl and sucrose but not NaCl medium. ATP-induced ethidium$^+$ uptake in a concentration dependant manner with a maximal response at 100 $\mu$M and with an EC$_{50}$ of 8.5 $\mu$M.
which is ~ 10 fold lower than that reported for recombinant P2X7-induced cation fluxes. Moreover, the non-P2X7 agonist, 5’-O-[3-thiotriphosphate] (ATP-γ-S) induced ethidium⁺ uptake in KG-1 cells. The P2X7 antagonists, KN-62, AZ10606120 and A-438079, each impaired ATP-induced ethidium⁺ uptake. ATP induced KG-1 cell death and morphological analysis indicated that this process was characteristic of apoptosis. ATP-induced apoptosis was impaired by KN-62 and AZ10606120. Collectively, these results indicate that KG-1 cells express functional P2X7 with an atypical pharmacological profile.
Chapter 1: Introduction

1.1 Purinergic Signalling

Adenosine 5'-triphosphate (ATP) is the main source of free intracellular energy in cells; however it is now widely accepted that ATP also functions as an extracellular signalling molecule. The role of ATP as an extracellular signalling molecule was first established by Burnstock (1972) in neurotransmission which gave rise to the term purinergic signalling. ATP is secreted into the extracellular environment under conditions of tissue stress, inflammation or, infection or during neurotransmitter release to function in an autocrine or paracrine fashion (Lazarowski et al., 2003). In the extracellular environment, ATP activates a group of cell-surface nucleotide or purinergic receptors, called P2X and P2Y receptors.

The P2X purinergic receptors are a trimeric, ligand-gated cation channels, which upon activation by ATP allows the influx of Na$^+$ and Ca$^{2+}$ ions, and an efflux of K$^+$. Seven subtypes of P2X subunits (P2X1-P2X7) have been cloned (North, 2002). P2Y receptors are G-protein-coupled metabotropic receptors, activated by ATP, ADP, uridine 5'-diphosphate (UDP) and uridine 5'-triphosphate (UTP). Eight P2Y receptor subtypes have been cloned (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14) (von Kugelgen, 2006). The role of P2X and P2Y receptors in physiology is gaining importance as they are widely distributed on a large array of cell types and induce important events in cells including proliferation, differentiation, apoptosis or necrosis, and release of inflammatory and tissue regenerating molecules. However the precise mechanisms of these receptors are yet to be fully characterised (Burnstock, 2007).
1.2 The P2X7 Receptor

1.2.1 P2X7 subunit

The P2X7 subunit is a 595 amino acid sequence consisting of an intracellular N-terminus, two hydrophobic transmembrane domains, an extracellular loop and an intracellular C-terminus (North, 2002) (Fig.1). The N- and C-termini of P2X7 comprise amino acids 1-25 and 356-595 respectively and form intracellular complexes with several membrane proteins including ß-actin, receptor-like tyrosine phosphatase and heat shock proteins (Kim et al., 2001). In addition, the C-terminus is essential for pore formation and receptor trafficking (Smart et al., 2003; Suprenant et al., 1996). It has also shown to stabilize the expression of P2X7 receptor in the membrane (Boldt et al., 2003; Denlinger et al., 2003; Feng et al., 2006). The first and second transmembrane domains comprise amino acids 26-46 and 335-355 respectively. The extracellular loop (amino acids 47-334) comprises the main binding site of ATP (North, 2002) and also contains five N-glycosylation sites (Zhou et al., 2009), of which Asn\textsuperscript{187}, Asn\textsuperscript{213} and Asn\textsuperscript{241} have been proposed to contribute towards P2X7 function (Feng et al., 2006; Gorodeski, 2009). The P2X7 subunits are assembled as a trimeric molecule to form functional P2X7 receptors (Nicke, 2008).

1.2.2 P2X7 pore formation

The activation of the P2X7 receptor by ATP or the most potent P2X7 agonist, 2(3-)O-(4-benzoylbenzoyl) adenosine 5\textsuperscript{\textprime}triphosphate (BzATP) causes a rapid influx of Ca\textsuperscript{2+} and Na\textsuperscript{+}, and an efflux of K\textsuperscript{+} by the opening of a non-selective cation channel, which is similar to that observed in other P2X receptors. However, the P2X7 receptor is a unique member of the P2X family in that, prolonged exposure to its agonists causes the formation of a pore permeable to organic molecules up to 900 Da in size (Burnstock,
Fluorescent dyes such as ethidium\(^+\) (314 Da) and YO-PRO-1\(^2+\) (375 Da) are routinely used to assay the pore properties of this receptor (Burnstock, 2007). Initially it was implied that the dye uptake took place via the ion channel that dilated into the pore over prolonged exposure to the agonist (North, 2002). More recent evidence however suggests the activation of a distinct hemichannel, pannexin-1, and subsequent interaction with P2X7 to form the dye uptake pathway (Pelegrin and Suprenant, 2006). Pannexins are a three-member family (pannexin 1-3) of mammalian membrane proteins that form gap-junction like connections, thus it remains unclear how pannexin-1 allows the intracellular passage of organic dyes (Pelegrin and Suprenant, 2006). Pannexin-1 also appears to be essential for the P2X7-induced maturation and release of interlukin (IL)-1\(\beta\) from macrophages (Pelegrin and Suprenant, 2006). However the role of pannexin-1 in P2X7 pore formation is still controversial and requires verification by others.

**Fig.1.1** Schematic representation of the P2X7 subunit. The P2X7 protein is a 595 amino acid sequence consisting of an intracellular N-terminus (blue), two hydrophobic transmembrane domains (green), an extracellular loop (black) and an intracellular C-terminus (red). Adapted from Kim *et al* (2001)
1.2.3 P2X7 polymorphisms

The human P2RX7 gene is localized in a 55-kb region of chromosome 12q34 and comprises 13 exons (Buell et al., 1998). The P2RX7 gene is highly polymorphic with more than 686 single-nucleotide polymorphisms (SNPs) (Sun et al., 2010). However, the functional effects of most SNPs are unclear. Most of the SNPs (G150R, R307Q, T357S, E496A, and I568N) characterised to date have been shown to cause loss-of-function (Gu et al., 2001, 2004; Shemon et al., 2006 Stokes et al., 2010; Wiley et al., 2003). Cabrini et al. (2005) identified the first gain-of-function SNP (H155Y). Other gain-of-function SNPs (A166G and A348T) have also been described (Denlinger et al., 2006; Stokes et al., 2010; Sun et al., 2010). Genetic studies have implicated the E496A SNP with increased susceptibility to chronic lymphocytic leukemia and tuberculosis (Fernando et al., 2007; Saunders et al., 2003; Wiley et al., 2002; Dao-Ung et al., 2004). The Q460R SNP has been associated with increased susceptibility to unipolar and bipolar mood disorders (Barden et al., 2006; Macquillin et al., 2008); however this SNP does not alter function (Fuller et al., 2009). Stokes et al (2010) identified five variants of a 4-SNP haplotype block located within exons 11 to 13 of the human P2RX7 gene. Of note, the P2X7-4 variant contained both the Q460R and A348T SNPs suggesting that the association of the Q460R with the gain-of-function A348T SNP may account for the association of Q460R with mood disorders (Stokes et al., 2010). These findings suggest that future genetic association studies of P2X7 SNPs and disease should consider P2RX7 haplotypes rather than single SNPs.
1.2.4 Splice variants of P2X7

P2X7 function involves the tri-oligomerization of the full-length receptor, influenced by the presence of monomer units and truncated variants of the P2X7 receptor (Le et al., 1998; Lewis et al., 1995; MacKenzie et al., 1999; Torres et al., 1999; Virginio et al., 1998). A P2X7 splice variant, in which the transmembrane 1 is deleted, does not generate functional receptors upon heterologous expression (Cheewatrakoolpong et al., 2005), whereas deletion of the cytoplasmic tail prevents dye uptake and pro-apoptotic effects (Suprenant et al., 1996). Nine truncated variants of the human P2X7 receptor (P2X7-b to P2X7-j) via alternative splicing were originally identified (Cheewatrakoolpong et al., 2005; Feng et al., 2006). Only one, the P2X7-b variant, out of the nine variants was found to be partially functional. This variant, expressed in most human tissues, was found to contain P2X7 channel but not pore activity (Cheewatrakoolpong et al., 2005). Whether the P2X7-b variant interacts with the full-length P2X7 to form heteromeric channels remains unknown. In contrast, the P2X7-j variant is found in malignant epithelial cells, hetero-oligomerizes with full-length P2X7 to form P2X7 heteromers that completely lack channel or pore activity, and fail to mediate P2X7-mediated apoptosis (Feng et al., 2006). The P2X7-j variant lacks the distal third of the extracellular loop, the second transmembrane domain, and the entire C-terminus (Feng et al., 2006). Recently, Nicke et al (2009) identified and characterised a novel P2X7 variant, P2X7-k, highly expressed in rodent liver and spleen. This variant has an alternative N-terminus and transmembrane domain 1 and has an 8-fold higher sensitivity to BzATP compared to the wild-type P2X7 (P2X7-a) and high levels of organic cation uptake. A greater understanding of this variant will provide a valuable tool in elucidating the mechanism involved in P2X7-mediated pore formation.
1.2.5 P2X7/P2X4 heteromers

Structural and functional interactions between the P2X7 and P2X4 receptors has gained much interest since the P2X4 receptor is nearly 40% more homologous to the P2X7 receptor than other P2X receptors, and the P2RX4 gene is located 130 kb downstream of the P2RX7 gene (Buell et al., 1998). The P2X7/P2X4 heterotrimer has been reportedly found in macrophages, monocytes, microglia, and endothelial and epithelial cells (Guo et al., 2007). However, Nicke (2008) showed that heterotrimerization of the P2X7 and P2X4 receptors results in unstable complexes and thus P2X7/P2X4 heteromers are unlikely to form a dominant subtype in tissues.

1.2.6 P2X7 Agonists

Each P2X receptor displays a unique pharmacological profile (Table 1). The P2X7 receptor is distinct from the other P2X members requiring 10- to 100-fold higher ATP concentrations for its activation compared to other P2X receptors (North and Suprenant, 2000). The half-effective concentration (EC50) of ATP required to activate the P2X7 receptor is ~100 µM, with maximal activation obtained at 300 µM ATP (North and Suprenant, 2000). The ATP analog, BzATP, is 10-30 times more potent than ATP in activating P2X7, although BzATP is not specific to P2X7 as it also activates P2X1 and P2X3 (North, 2002). Agonist potency at the P2X7 receptor is ranked in order of BzATP > ATP >> 2-methylthiodenosine 5′triphosphate (2MeSATP) > adenosine 5′O-(3-thio) triphosphate (ATPγS) >>> ⍥-methylene adenosine 5′triphosphate (⍥meATP) > ADP > UTP (Suprenant et al., 1996). The presence of µM concentrations of ATP has not been observed in vivo, but data with P2X7 knock-out indicate that ATP can be released to activate P2X7 in vivo at least under particular pathophysiological conditions (Labasi et al., 2002; Chessel et al., 2005).
1.2.7 P2X7 Antagonists

A number of specific P2X7 antagonists are now commonly available. The isoquinoline sulfonamide 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), is a potent, non-competitive, human P2X7 antagonist (Gargett and Wiley, 1997) which has been used for more than a decade. KN-62 however also inhibits calmodulin-dependant protein kinase II (Chessell et al., 1998) complicating its use when studying events downstream of P2X7 activation. KN-62 is inactive at the rat P2X7 homolog (Jiang et al., 2000). The tetrazolylmethylpyridine derivative, A-438079 (Nelson et al., 2006), and the adamantyl derivative, AZ10606120 (Michel et al., 2008), are also potent, selective, and competitive P2X7 antagonists active at both the human and rat P2X7 receptor. A biphenyl derivative, AZ11645373, also selectively antagonizes the human P2X7 receptor, but is inactive at the rat P2X7 (North, 2002) and other P2X subtypes (Stokes et al., 2006). Suramin, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and oxidised ATP have also been commonly used antagonists of the human and rat P2X7 receptor, however their specificity has been questioned (Di Virgillio, 2003). Thus, given the availability of KN-62, A-438079 and the AZ11645373, the use of suramin, PPADS and oxidised ATP should be discouraged.

In addition to the above compounds, the P2X7 receptor is also impaired by various extracellular ions. Virginio et al. (1997) carried out a comprehensive study of the functional inhibition of P2X7 by divalent cations. They showed that Ca^{2+}, Mg^{2+}, Zn^{2+} and Cu^{2+} all strongly inhibited both P2X7-mediated currents and YO-PRO-1^{2+} dye uptake. The divalent cations showed the same rank order of potency when inhibiting the P2X7-mediated currents and dye uptake with Cu^{2+} > Cd^{2+} ~ Zn^{2+} > Ni^{2+} >> Mg^{2+} ~ Co^{2+} > Mn^{2+} > Ca^{2+} = Ba^{2+} >> Sr^{2+}. Removal of Ca^{2+} and Mg^{2+} ions from media is
thought to increase the availability of ATP\textsuperscript{4+} required for P2X7 activation (North, 2002), however, the inhibitory action of the other divalent cations remains unknown. Extracellular Na\textsuperscript{2+} ions can also impair P2X7 receptor function. Michel et al. (1999) have shown that the potency of BzATP for human P2X7 is approximately 20-fold higher in sucrose medium (containing nominal amounts of Na\textsuperscript{2+}) compared to NaCl medium. Similarly, maximal response and efflux rates of P2X7 to ATP and BzATP are higher in KCl medium compared to NaCl medium (Wiley et al., 1993; Stevenson et al., 2009).
Table-1.1 Human P2X subtypes.

<table>
<thead>
<tr>
<th>P2X subtypes</th>
<th>Gene location</th>
<th>amino acids (µM)</th>
<th>ATP EC$_{50}$ (µM)</th>
<th>Rank agonist profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X1</td>
<td>17p13.3</td>
<td>399</td>
<td>1</td>
<td>2MeSATP &gt; αβmeATP ~ BzATP &gt; ATP &gt; ADP</td>
</tr>
<tr>
<td>P2X2</td>
<td>12q24.33</td>
<td>471</td>
<td>10</td>
<td>ADP ~ 2MeSATP &gt; ATP &gt; BzATP &gt;&gt; αβmeATP</td>
</tr>
<tr>
<td>P2X3</td>
<td>11q12</td>
<td>397</td>
<td>1</td>
<td>2MeSATP ~ 2MeSATP &gt; ATP &gt; ADP</td>
</tr>
<tr>
<td>P2X4</td>
<td>12q24.32</td>
<td>388</td>
<td>10</td>
<td>2MeSATP &gt;&gt;&gt; ATP &gt;&gt; αβmeATP</td>
</tr>
<tr>
<td>P2X5</td>
<td>17p13.3</td>
<td>422</td>
<td>10</td>
<td>ADP &gt;&gt; ATP</td>
</tr>
<tr>
<td>P2X6</td>
<td>22q11.21</td>
<td>431</td>
<td>6</td>
<td>ATP &gt; αβmeATP</td>
</tr>
<tr>
<td>P2X7</td>
<td>12q24</td>
<td>595</td>
<td>100</td>
<td>2MeSATP &gt; ATP$_{2S}$ &gt;&gt;&gt; αβmeATP &gt; ADP &gt; UTP</td>
</tr>
</tbody>
</table>

*Abbreviations:* 2(3'O-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP), 2-methylthio adenosine triphosphate (2MeSATP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), adenosine 5'-O-[3-thiotriphosphate] (ATP-$\gamma$S), αβ-methyl ATP (αβmeATP), uridine 5'-triphosphate (UTP). Table adapted from North, 2002.
1.2.8 Distribution of P2X7

P2X7 receptors have been identified on a variety of cell types by electrophysiology techniques and other functional assays, reverse transcription polymerase chain reaction (RT-PCR), immunocytochemistry, immunoblotting and gene knock-out approaches. P2X7 receptors are highly expressed on cells from the hematopoietic lineages, such as erythrocytes, T- and B-lymphocytes, eosinophils, mast cells, monocytes, dendritic cells and macrophages (Sluyter et al., 2001, 2004, 2007; Gu et al., 2000; Adinofi et al., 2002; Suh et al., 2001; Bulanova et al., 2005; Gudipaty et al. 2001; Wareham et al., 2009; Zhang et al., 2005). P2X7 receptors are also found abundantly in brain glial cells (microglia, astrocytes, and Muller cells), bone cells (osteoblasts, osteoclasts, and osteocytes), epithelial cells and endothelial cells (Gartland et al., 2003; Bianco et al., 2005, 2006; Sugiyama et al., 2005; Li et al., 2005; Pennolazzi et al., 2005; Li et al., 2003). P2X7 receptors have also been reported in central and spinal cord neurons (Miras-Portugal et al., 2003; Leon et al., 2006; Marin-Garcia et al., 2008), however the expression of P2X7 in these tissues remains controversial (Sim et al., 2004). Expression of P2X7 receptors has also been found in cells originating from the small intestine, kidney, urinary tract, uterus, and liver (Hillman et al., 2005; Koshi et al., 2005; Emmett et al., 2008). P2X7 receptors have also been observed in a variety of malignant cell lines (White and Burnstock, 2006) including myeloid leukemic and epithelial cells, and are disclosed in greater detail in Section 1.3, 1.4, 1.5.

1.2.9 P2X7 activation induces downstream signalling events

P2X7 receptor activation induces a number of downstream signalling events including the release of pro-inflammatory cytokines and metalloproteases, reactive
oxygen and nitrogen species formation, shedding of cell adhesion molecules, killing of intracellular pathogens, and cell death and proliferation (Table 1.2). Of these the maturation and release of interleukin (IL)-1β is the best described downstream signalling event of P2X7 activation (Mariathasan and Monack, 2007; Ferrari et al., 2006) and is discussed in further detail below. P2X7 activation can also induce cell death (Adinolfi et al., 2005), but under some conditions and in certain cell types P2X7 activation may, conversely, induce cell proliferation (Di Virgilio, 2009). As a result of this dual role of P2X7 in cell survival, this receptor is attracting interest for its growth inhibitory and growth promoting roles in normal and malignant cell types (White and Burnstock, 2006; Di Virgilio, 2009). Data supporting a role for P2X7 activation in cell death and proliferation is discussed further below.

**P2X7 activation induces IL-1β maturation and release**

IL-1β is a pro-inflammatory cytokine predominantly released from monocytes, macrophages and dendritic cells (Dinarello, 2005). Activation of P2X7 on these cells leads to the maturation and release of this cytokine (Mariathasan and Monack, 2007; Ferrari et al., 2006) (Fig. 1.2). IL-1β is constitutively present in macrophages, but is normally absent in monocytes and dendritic cells. Stimulation of each of these cell types with pathogen-associated molecular pattern molecules, such as lipopolysaccharide (LPS), activate the nuclear factor-kappa beta (NF-κB) cascade to induce synthesis of pro-IL-1β, but are unable to cause the functional maturation and release of this cytokine (Dinarello, 2005; Ferrari et al., 2006; Mariathasan et al., 2006). A secondary stimulus, such as ATP, is required for efficient processing and release of bioactive IL-1β (Perregaux and Gabel, 1994, 1998). Activation of the P2X7 receptor by ATP leads to the loss of cytoplasmic K⁺, and the assembly and activation of the NALP3 (NLRP3)
inflammasome, a multi-protein complex that recruits pro-caspase-1 and induces its proteolytic activation (Ferrari et al., 2006; Mariathasan et al., 2006). Pannexin-1 is also critical for NALP3 inflammasome, and P2X7-mediated IL-1β maturation and release (Pelegrin et al., 2008; Pelegrin and Surprenant, 2006, 2007). These studies demonstrated that pannexin-1 is required for caspase-1 activation in response to ATP suggesting a direct functional link between dye uptake, activation of caspase-1, and IL-1β maturation and release. However the exact role of pannexin-1 in the IL-1β signalling cascade including the NALP3 inflammasome remains to be fully determined (Mariathasan and Monack, 2007). In addition to pannexin-1, P2X7-induced IL-1β maturation and release is dependent on the activation of a Ca^{2+}-independent phospholipase A₂ (iPLA₂) following the decrease in cytoplasmic K⁺ concentration (Andrei et al., 2004), however a link between the PLA₂ and the NALP3 inflammasome is yet to be determined.

The mechanism of IL-1β release is less clear. Studies have shown that the activation of the P2X7 receptor induces shedding of microvesicles containing mature IL-1β from the human monocytic cell line, THP-1 (MacKenzie et al., 2001) and monocyte-derived dendritic cells (Pizzirani et al., 2007). However another study shows that P2X7 activation causes the co-localisation of pro-IL-1β and caspase-1 in secretory lysosomes in primary human monocytes, where it matures before secretion by exocytosis (Andrei et al., 2004). Collectively, this data suggests that the maturation and release of IL-1β varies according to cell type, state of differentiation or conditions used for P2X7 receptor activation.
Fig. 1.2 P2X7 activation induces IL-1β maturation and release. Activation of P2X7 by ATP results in K⁺ efflux and the activation of pannexin-1 to activate the NALP3 (nucleotide-binding domain and leucine-rich repeat containing a pyrin domain) complex containing the adaptor molecule ASC (apoptosis-associated speck-like protein containing a CARD) and cardinal. This complex aggregates pro-caspase-1 molecules to form an inflammasome complex that facilitates pro-caspase-1 activation and subsequent pro-IL-1β processing to its mature IL-1β form. Loss of cytoplasmic K⁺ by P2X7 also activates a calcium-independent phospholipase A₂ (iPLA₂) to activate pro-caspase-1 and the processing of pro-IL-1β to its mature form. Figure adapted from Pelegrin and Surprenant (2009)
**P2X7 activation induces cell death**

It has been known for close to three decades that extracellular ATP can induce cell death by either apoptosis or necrosis (Di Virgilio *et al.*, 1998). However despite many reports of ATP-induced cell death, and that this effect is often induced by P2X7 activation (Adinolfi *et al.*, 2005), the mechanisms involved in P2X7-induced apoptosis or necrosis remain poorly defined. This is attributed in part to the limited use of experimental parameters to clearly define cells as having undergone apoptosis or necrosis (Kroemer *et al.*, 1998). Moreover, the downstream pathways involved in P2X7-induced cell death appear to differ between various cell types and experimental conditions making comparisons between individual studies difficult. In 1999, Ferrari and colleagues provided some of the earliest evidence that P2X7 induces apoptosis. In this study, high concentrations of ATP (3 mM) induced condensation of nuclei, DNA fragmentation and caspase-1, -3 and -8 activation in mouse microglial N3 and N8 cells (Ferrari *et al.*, 1999). Similarly, high concentrations of ATP (5 mM) induced apoptosis in mouse BAC1 macrophages, as evidenced by DNA fragmentation and caspase-3 activation (Humphreys *et al.*, 2000). Moreover, this process was impaired by KN-62 directly implicating P2X7 in this ATP-induced apoptosis. Similar evidence for P2X7 activation in ATP-induced apoptosis has also been observed in mouse RAW264.7 macrophages (Noguchi *et al.*, 2008). Recently, Di Virgilio (2009) proposed a mechanism by which P2X7 activation induces apoptosis (Fig. 1.3). In this model, sustained P2X7 activation by high concentrations of ATP causes a collapse of the mitochondrial membrane potential to activate the caspase pathway to induce apoptosis. Further roles for P2X7 in ATP-induced cell death in other cell types such as epithelial cells, as well as myeloid and lymphoid leukemic cells is discussed further below.
**P2X7 activation induces cell proliferation**

Activation of the P2X7 receptor can induce the proliferation of certain cell types including P2X7-transfected leukemic, K562 and LG14 cell lines, and normal and leukemic T- and B-lymphocytes (Adinolfi et al., 2002; Baricordi et al., 1996, 1999; Budagion et al., 2003; Yip et al., 2009). The molecular basis underlying this process is not well understood. In an attempt to understand the mechanism involved in P2X7-mediated cell proliferation, Adinolfi et al (2005) showed that HEK-293 cells transfected with the P2X7 receptor resulted in higher resting concentrations of mitochondrial Ca\(^{2+}\) and the subsequent release of large amounts of Ca\(^{2+}\) from intracellular stores. In turn, this Ca\(^{2+}\) increase stimulated NADH synthesis and ATP production via oxidative phosphorylation. This increased intracellular ATP content facilitated cell survival and growth. In a recent study by the same group, P2X7 expression in transfected HEK-293 cells increased intracellular Ca\(^{2+}\) by disrupting endoplasmic reticulum function to activate the nuclear factor of activated T-cells (NFATc1) to enhance cell survival and protect the cells from apoptosis (Adinolfi et al., 2009). This mechanism of P2X7-induced cell proliferation is shown in Fig. 1.

Other mechanisms of P2X7-induced proliferation have also been described in chronic lymphocytic leukemia (CLL) B-lymphocytes, and normal and malignant T-lymphocytes and are discussed further below. Despite the varying mechanisms involved in P2X7-induced cell death and proliferation, one common feature of P2X7-induced cell proliferation is that, this process is stimulated by low amounts or basal amounts of ATP added to or released from the cells respectively (Di Virgilio, 2009), in contrast to P2X7 induced cell death, which requires at least 10-fold higher concentrations of ATP (Adinolfi et al., 2005).
**Fig. 1.3** Schematic representation of P2X7 as a growth-promoting and death-inducing receptor. Basal activation of the P2X7 receptor leads to a moderate increase in cytoplasmic Ca\(^{2+}\), which causes an elevation of intramitochondrial Ca\(^{2+}\) that stimulates NADH synthesis and ATP production via oxidative phosphorylation. Increased cellular ATP content then facilitates cell survival and growth. Prolonged activation of the P2X7 receptor causes uncontrolled influx of Ca\(^{2+}\), which triggers loss of mitochondrial membrane potential followed by activation of caspase 9/7/3-mediated cell death. Figure adapted from Di Virgilio et al (2009).

**P2X7 activation induces reactive oxygen species production**

Several studies have indicated a role for P2X7-mediated reactive oxygen species (ROS) production. Lenertz et al (2009) showed that P2X7 mediates ROS production in primary human monocytes and RAW264.7 macrophages, and that generation of ROS most likely involves activation of ERK1/2 and the NADPH oxidase complex. These
findings were consistent with that of Noguchi et al. (2008) who demonstrated that P2X7-induced ROS production in RAW264.7 macrophages is sensitive to the NADPH oxidase inhibitor apocynin and can be prevented by reducing the expression of the NADPH subunit gp91phox in these cells. Moreover, this study demonstrated that ROS formation plays a role in P2X7-induced apoptosis of RAW264.7 macrophages; however, a role for ROS in P2X7-induced apoptosis has not been reported by others.
<table>
<thead>
<tr>
<th>Cell type(s)</th>
<th>Signalling mechanism(s)</th>
<th>Cellular function(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendritic cells, macrophages, microglia, monocytes</td>
<td>K⁺ efflux, NALP3</td>
<td>IL-1β and IL-18</td>
<td>Perregaux and Gabel, 1994; Andrei et al., 2004; Hewinson, et al., 2008; Mariathasan et al., 2006; Pelegrin and Surprenant, 2006</td>
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<td>T- and B-lymphocytes, microglia, erythroleukemic cells</td>
<td>Ca²⁺ influx, NFAT, p38, pannexin-1</td>
<td>Cell proliferation</td>
<td>Adinolfi et al., 2006; Baricordi et al., 1999; Ferrari et al., 1999; Loomis et al., 2003; Yip et al., 2009</td>
</tr>
<tr>
<td>B-lymphocytes, macrophages, microglia</td>
<td>JNK, p38, NADPH oxidase, caspase-3 and -8</td>
<td>Cell death, ROS formation</td>
<td>Ferrari et al., 1999; Humphreys et al., 2000; Noguchi et al., 2008; Lenertz et al., 2009; Noguchi et al., 2008; Pfeiffer, et al., 2007</td>
</tr>
<tr>
<td>Macrophages</td>
<td>JNK, p38, NADPH oxidase</td>
<td>CD23 shedding</td>
<td>Gu et al., 1998; Le Gall et al., 2009; Sluyter and</td>
</tr>
<tr>
<td>Cell Type</td>
<td>Trigger</td>
<td>Event</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>------------------------------</td>
<td>------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>B- and T- lymphocytes, monocytes</td>
<td>ADAM-17</td>
<td>CD62L shedding</td>
<td>Gu et al., 1998; Le Gall et al., 2009; Sluyter et al., 2004; Seman et al., 2003</td>
</tr>
<tr>
<td>B- and T- lymphocytes, monocytes</td>
<td>Ca(^2+) influx</td>
<td>MMP-9 release</td>
<td>Gu and Wiley, 2006</td>
</tr>
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<td>P2X7-transfected fibroblasts, macrophages, osteoblasts</td>
<td>p38, Rho, PLD, PLA(_2), LPA</td>
<td>Actin reorganization and membrane blebbing</td>
<td>Morelli et al., 2003; Pfeiffer, et al., 2004; Verhoef et al., 2003</td>
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<td>Endothelial cells, erythroleukemic cells, dendritic cells, macrophages, microglia</td>
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<td>Microvesicle release</td>
<td>Baroni et al., 2007; Bianco et al., 2005; Constantinescu et al., 2010; Wilson et al., 2005</td>
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<tr>
<td>Dendritic cells</td>
<td>NALP3</td>
<td>Exosome release</td>
<td>Qu et al., 2009</td>
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<tr>
<td>Macrophages</td>
<td>Ca(^2+) influx, PLD, phagolysosome</td>
<td>Killing of intracellular mycobacteria</td>
<td>Kusner and Adams, 2001; Stober et al., 2003</td>
</tr>
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</table>
Abbreviations: IL, interleukin; JNK, Jun N-terminal Kinase; LPA, lysophosphatidic acid; PLA_2, phospholipase A_2; PLD, phospholipase D; RONS, reactive oxygen and nitrogen reactive species; ROS, reactive oxygen species
1.3 Role of P2X7 in THP-1 Monocytes

Following on from the pioneering work of Blanchard (Spranzi et al., 1993) and Dubyak (Humphreys and Dubyak, 1996, 1998; Humphreys et al., 1998) and their colleagues, the human leukemic monocytic cell line THP-1 has become a well-established model to study the expression and function of P2X7. THP-1 cells express no to low amounts of P2X7, however the expression of this receptor can be induced following differentiation with IFN-γ and LPS, or to a lesser extent with 12-myristate 13-acetate (PMA) (Humphreys and Dubyak, 1998). Similarly, P2X7 expression and function is upregulated upon differentiation of monocytes to macrophages (Hickman et al., 1994). Moreover, pro-inflammatory molecules such as interferon-γ (IFN)-γ, tumour necrosis factor-α and lipopolysaccharide (LPS) can up-regulate P2X7 expression and function in monocytes/macrophages (Blanchard et al., 1991; Buell et al., 1998; Gudipaty et al., 2001). Thus, due to the effects of P2X7 in monocytes and macrophages, the THP-1 cell line is considered as a suitable model to study P2X7 in monocytes/macrophage biology. Moreover, THP-1 cells differentiated with IFN-γ and LPS demonstrate characteristics of primary macrophages including plastic adherence (Humphreys and Dubyak, 1998), phagocytosis of latex beads (Gu et al., 2010) and killing of intracellular mycobacteria (Kusner et al., 2000)

Activation of P2X7 in differentiated THP-1 cells results in number of downstream signalling events including: cation fluxes (Humphreys and Dubyak, 1998); cell lysis (Spranzi et al., 1993); activation of phospholipase D (Humphreys and Dubyak, 1996), mitogen-activated protein kinases (Aga et al., 2002) and caspase-1 (Verhoef et al., 2005); nuclear translocation of NF-κB (Aga et al., 2002); dissociation of nonmuscle myosin from the P2X7 complex (Gu et al., 2009) and subsequent inhibition of
phagocytosis (Gu et al., 2010); release of mature IL-1β (Grahames et al., 1999) and IL-18 (Bachmann et al., 2006); shedding of microvesicles (MacKenzie et al., 2001); and killing of intracellular mycobacteria (Kusner et al., 2000). THP-1 monocytes have also been widely used to screen a number of potential P2X7 antagonists leading to the development of some highly specific and potent P2X7 antagonists (Nelson et al., 2006; Stokes et al., 2006). Finally, this cell line was used to establish the blocking action of the only available human anti-P2X7 monoclonal antibody (mAb) (Buell et al., 1998).
1.4 Role of P2X7 in Epithelial Cancer

The P2X7 receptor is thought to play an important physiological role in the physiology and pathophysiology of normal and malignant epithelial cells (Gorodeski, 2009). Moreover, ATP is present in the extracellular milieu of epithelial tissues at concentrations sufficient to activate the P2X7 receptor (Gorodeski, 2009). Most data supporting a role for P2X7 in epithelial tissues is based on studies with colon, skin and cervical epithelial cells.

A number of studies have examined the expression and function of the P2X7 receptor in normal and malignant epithelial colon cells, although direct evidence for this receptor in these cells is limited. Selzner et al (2004) observed that extracellular ATP induced apoptosis of the human epithelial colon cancer cell lines, SW403, HCT 116 and Colo. Moreover, this ATP-induced apoptosis was mediated by the release of cytochrome $c$ and subsequent activation of caspase 3. Finally, the authors attributed this effect to P2X7 receptor activation, as the various cell lines expressed this receptor, however direct pharmacological evidence was lacking. Following on from the observations, Coutinho-Silva et al (2005) demonstrated the presence of low levels of P2X7 mRNA and P2X7-induced Ca$^{2+}$ fluxes in the human epithelial colon carcinoma cell lines, HCT-8 and Caco-2. A dual role for P2 receptor signalling was implicated in these cell lines, as ATP induced cell proliferation at low concentrations, but at higher concentrations induced apoptosis. Moreover, oxidised ATP partially blocked this ATP-induced apoptosis which lead the authors to conclude that this process was mediated by P2X7, however direct pharmacological evidence using more specific P2X7 antagonists was lacking. Subsequently, the authors demonstrated that IFN-$\gamma$ increased the susceptibility of HCT-8 cells to ATP-induced apoptosis, however the effect of IFN-$\gamma$ on
P2X7 mRNA or protein was not examined (Welter-Stahl et al., 2009). Moreover, direct pharmacological evidence for P2X7 in mediating ATP-induced apoptosis was again lacking. Finally, Li et al (2009) have shown that the level of P2X7 expression is similar in normal and cancer colon cells, which is in contrast to other epithelial cell types, including those from the cervix and skin, where P2X7 is approximately 2-fold lower in the malignant cells compared to normal cells. The significance of these latter findings is not well understood.

A number of groups have detected the presence of functional P2X7 receptors in normal and malignant skin epithelial cells (Holzer and Granstein, 2004; White and Burnstock, 2006). A role for skin epidermal P2X7 in inflammatory and immune processes has been suggested (Georgiou et al., 2005), but direct evidence was lacking at the time. Subsequently, others have shown that P2X7 activation can impair the release of the chemokine CXCL16 from cultured human normal keratinocytes (Pastore et al., 2007), while others have reported that treatment of these cells with BzATP induces IL-6 release (Inoue et al., 2007). More, recently Fu et al. (2009) have shown that P2X7 activation induces apoptosis of cultured normal mouse keratinocytes. Furthermore, this process was dependent on a Ca\(^{2+}\) influx and the subsequent activation of caspase-9 (or mitochondrial) apoptotic pathway. Of note, this paper went on to demonstrate that P2X7 activation could prevent the formation of skin papillomas and cancer in mice following treatment with chemical carcinogens supporting a role for P2X7 activation in vivo.

A series of studies from the laboratory of Gorodeski provide evidence for a role for P2X7 in normal and malignant cervical epithelial cells. BzATP induces cell death in both normal human ectocervical epithelial cells and the human epithelial ecto-cervical CaSki cell line in a Ca\(^{2+}\)- and caspase-9-dependent manner, suggesting that P2X7
activation induces the apoptotic mitochondrial pathway in these cells (Wang et al., 2004). A direct role for P2X7 in this process was established when it was shown that co-expression of the P2X7-j variant (Section 1.1.2) with full-length P2X7 blocked BzATP-induced apoptosis in these cells (Feng et al., 2006). Other studies demonstrated that both estrogen and epinephrine can impair the P2X7-induced apoptosis of human cervical epithelial cells (Gorodeski, 2004; Wang et al., 2005). Estrogen impaired P2X7-induced apoptosis by decreasing Ca\(^{2+}\) influx and the subsequent increase in cytosolic Ca\(^{2+}\) increase via ATP-activated P2X7 pores (Gorodeski, 2004). These results suggest that estrogen may have an anti-apoptotic role within the cervix. The inhibitory effect of epinephrine was due to increased degradation of full-length P2X7 and the subsequent decrease of total P2X7 protein resulting in reduced P2X7-induced pore formation and apoptosis. Finally, Li et al. (2009) showed that the amounts of full-length P2X7 are lower in cervical and uterine epithelial cancer tissues than in corresponding normal tissues. Thus, tissue analysis of P2X7 mRNA and protein levels could be used as a novel biomarker to differentiate between normal and malignant cells in these epithelial tissues.
1.5 Role of P2X7 in Leukemia

Functional P2X7 receptors have been reported in various myeloid and lymphoid leukemias, as well as multiple myeloma. P2X7 has been identified in a number of myeloid leukemic cell lines, however evidence of this receptor in primary myeloid leukemias is lacking. In addition to THP-1 cells (Section 1.3), P2X7 mRNA has been observed in the human myeloid leukemic HL-60 and F-36P cell lines (Yoon et al., 2006). Moreover, ATP induced cell cycle arrest in these cell lines, but the identity of the P2 receptor was not established (Yoon et al., 2006). P2X7 mRNA and protein has also been observed in the human myeloid leukemic KG1a cell line, but lacked functional ATP-induced responses in NaCl medium (Zhang et al., 2004). However, subsequent studies of this cell line in sucrose medium have revealed that BzATP can induce Ca\textsuperscript{2+} fluxes, pore formation and cell death in this cell line (Zhang et al., 2009). Moreover, this latter study showed that oxidised ATP could impair these BzATP-induced responses, but other direct evidence for involvement of the P2X7 receptor was lacking. Finally, there are no reports as to whether the parental myeloid leukemic cell line, KG-1 (from which KG1a cells are derived) expresses functional P2X7.

P2X7 is present in malignant B-lymphocytes from patients with chronic lymphocyte leukaemia (CLL) (Gu et al., 2000; Wiley and Dubyak, 1989;). Activation of P2X7 in this cell type results in cell death (Adinolfi et al., 2002; Wiley et al., 2002). Conversely, a role for P2X7 in the proliferation of CLL B-lymphocytes has also been proposed since CLL B-lymphocytes from patients with more progressive forms of the disease have higher rates of cell proliferation, and correspondingly increased amounts of functional P2X7, compared to cells from patients with indolent forms of CLL (Adinolfi et al., 2002). This dual role of P2X7 in cell death and proliferation most likely reflects
differences in ATP concentrations (high versus low respectively) as discussed by Di Virgilio and Wiley (2002). P2X7 activation can also induce the rapid shedding of cell-surface CD23 (the low affinity IgE receptor) from CLL B-lymphocytes via stimulation of a membrane metalloprotease (Gu et al., 1998). Although the identity of this metalloprotease was not established, more recent data indicate a role for ADAM10 in the P2X7-induced shedding of CD23 from P2X7-transfected Chinese hamster ovary cells and murine B-lymphocytes (Le Gall et al., 2009). Whether P2X7 is present in other types of human B-lymphocyte malignancies, apart from CLL, is unknown. However Farrell (2008) showed that the human multiple myeloma RPMI 8226 cell line expresses functional P2X7, and that activation of this receptor induces cell death and the rapid shedding of CD23 from the surface of these cells. Thus, this cell line provides a useful model to study the role of human P2X7 in multiple myeloma and as a positive control for other P2X7 studies.

The physiological and pathophysiological role of P2X7 in normal and malignant B-lymphocytes is not fully understood. So far there have been no reports of defects in B-lymphocyte mediated responses in P2X7 knockout mice (Chessell et al., 2005; Solle et al., 2001). Moreover, defects in B-lymphocyte function have not been attributed to altered disease outcomes in models of inflammatory or immune-mediated disorders in these mice (Chen et al., 2006). Both enhanced P2X7-induced cell proliferation, due to high P2X7 amounts (Adinolfi et al., 2002), and defects in P2X7-induced cell death, due to the E496A loss-of-function polymorphism (Wiley et al., 2002; Thunberg et al., 2002) have been postulated to alter disease outcomes in CLL (Virgilio and Wiley, 2002), however neither hypothesis has been substantiated by additional data (Sellick et al., 2004). Moreover, the E496A polymorphism is not associated with disease outcomes in
multiple myeloma (Paneesha et al., 2006). However, it should be noted that this loss-of-function polymorphism reduces P2X7 function by approximately 50% in heterozygous dosage (Gu et al., 2001), but does not inhibit P2X7 channel function (Boldt et al., 2003) nor completely impairs P2X7 function in homozygous dosage (Sluyter et al., 2004). Thus, the possibility remains that P2X7 may still play a role in B-lymphocyte malignancies independently of this polymorphic variation.

P2X7 is also present in human leukemic Jurkat T cells. Yip et al (2009) have recently shown that T-cell receptor (TCR) activation in Jurkat T cells, as well as primary human CD4+ T-lymphocytes up-regulates P2X7 mRNA and triggers the release of ATP which feeds back on these cells to further induce TCR-mediated Ca^{2+} influx, NFAT activation and interleukin-2 production to drive cell proliferation. Moreover, they have shown that T-lymphocyte activation is impaired in C57BL/6 mice that express low levels of functional P2X7, compared to BALB/c mice, which express fully functional P2X7 receptors. Prior to these observations, Budagion et al (2003) showed that treatment of Jurkat T cells with millimolar concentrations of extracellular ATP resulted in the phosphorylation and activation of p56^{ck} kinase, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase and the subsequent production of IL-2 and cell proliferation. These effects were shown to be completely dependent upon the presence of extracellular Ca^{2+} ions in the culture medium and pharmacological data supported the role of P2X7 in these ATP-induced effects in Jurkat T cells. Finally, the P2X7 mRNA has also been reported in primary lymphoid malignancies (Zhang et al., 2004), however the authors failed to report if they originated from either B- or T-lymphoid cells.
1.6 Transforming growth factor-β1

Transforming growth factor-β1 (TGF-β1) is a pleiotropic cytokine involved in a variety of biological processes including inflammation and immunity, where it is involved in homeostasis and tolerance, as well as the initiation and resolution of the immune response (Li et al., 2006). TGF-β1 belongs to the TGF-β superfamily, which includes three isoforms of TGF-β (TGF-β1 to -β3) (Li et al., 2006). TGF-β is synthesized as a pre-pro-TGF-β precursor molecule which requires subsequent processing by a furin-like peptidase within the Golgi apparatus (Li et al., 2006). The process product is then released from the cell in a latent form, but requires additional processing by changes in pH or heat or by several proteases before it can bind to its respective receptor (Li et al., 2006). Active forms of TGF-β bind to the Type II TGF-β receptor. This receptor then recruits, phosphorylates and activates the Type I TGF-β receptor. The TGF-β receptor also forms a complex with activin receptor-like kinase 5 (ALK5) to help mediate cell signalling.

A number of mechanisms of TGF-β signalling have been proposed including Smad-dependent and independent signalling (Li et al., 2006). Smad-dependent signalling occurs as a result of signalling via phosphorylated ALK5 and the Type I TGF-β receptor following TGF-β binding. These phosphorylated molecules in turn, phosphorylate receptor-activated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5, and Smad8). Once phosphorylated, R-Smads associate with the co-mediator Smad, Smad4, and the heteromeric Smad complex then translocates to the nucleus. In the nucleus,
Smad complexes activate specific genes through cooperative interactions with other DNA-binding and co-activator (or co-repressor) proteins (Shi and Massague, 2003).

TGF-β1 is the predominant TGF-β isoform expressed in the immune system. TGF-β1 has been associated with both pro- and anti-inflammatory functions on monocytes/macrophages through multiple mechanisms. For instance, TGF-β1 can induce IL-1, IL-6 and matrix metalloproteinases (MMPs) release from monocytes, and can facilitate monocyte migration to sites of infection and inflammation (Han et al., 2000; Wahl et al., 1993). Conversely, TGF-β1 can down-regulate CD14 or MHC class II expression in IFN-γ- or LPS-treated macrophages respectively (Nandon et al., 1997). TGF-β1 can also down-modulate the expression and function of several other cell-surface receptors in other cell types including CD1d on dendritic cells (Ronger-Savle et al., 2005), FcεRI on mast cells (Gomez et al. 2005) and Fas on follicular dendritic cells (Park et al., 2005). Finally, data from our laboratory has shown that TGF-β1 can down-regulate P2X7 cell-surface expression and P2X7 function (ATP-induced ethidium+ uptake) in IFN-γ and or LPS-treated THP-1 monocytes (Tran, 2007; Georgiou and Sluyter, personal communication), however the mechanism of action of TGF-β1 in this process was not established. While other, data from our laboratory, has shown that monocyte-derived dendritic cells generated in the presence of TGF-β1 have decreased amounts of P2X7 function (ATP-induced ethidium+ uptake) compared to monocyte-derived dendritic cells generated in the absence of TGF-β1 (Georgiou et al., 2005). However, in contrast to THP-1 cells, this difference in dendritic cells correlated with differences in cell-surface CD39 (ecto-ATPDase) but not P2X7 expression (Georgiou et al., 2005). Whether TGF-β1 effects P2X7 in other cell types is unknown.
1.7 AIMS

This thesis aims to examine the expression and function of the P2X7 receptor in human malignant myeloid and epithelial cell lines. Specifically, the aims are to:

(1) Determine the mechanism by which TGF-β1 prevents the up-regulation of P2X7 expression and function in IFNγ and LPS-treated myeloid leukemic THP-1 cells;

(2) Confirm if the colon epithelial HCT-8 and Caco-2 cell lines express functional P2X7 receptors, and whether activation of this receptor induces cell death;

(3) Determine if the myeloid leukemic KG-1 cell lines expresses functional P2X7 receptors.
Chapter 2: Materials and Methods

2.1 Materials

RPMI-1640 medium (containing 10 mM HEPES), foetal calf serum (FCS; inactivated before use), 0.25% trypsin solution, Dulbecco's phosphate-buffered saline (PBS), L-glutamine, Superscript III One-Step RT-PCR System Platinum Taq DNA polymerase and penicillin-streptomycin were from Invitrogen Corporation (Auckland, New Zealand). Adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), 2(3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP), uridine 5'-triphosphate (UTP), ethidium bromide, lipopolysaccharide (LPS; Escherichia coli 055:B5), non-essential amino acids, 6-aminohexanoic acid, propidium iodide, trypan blue solution and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich (St. Louis, Missouri, U.S.A.). Stocks of ATP (100 mM) were prepared in double distilled water, the pH adjusted to 7.5 with 1 mM NaOH, and stored in aliquots at -80°C. YO-PRO-1 was from Molecular Probes (Invitrogen, Eugene, OR, U.S.A.). Dimethyl sulphoxide (DMSO), n-dodecyl β-D-maltoside, phenylmethylsulfonyl fluoride (PMSF), 2-mercaptoethanol, Tween-20, Triton X-100, sodium dodecyl sulphate (SDS) and other general reagent grade chemicals were obtained from Amresco (Solon, Ohio, U.S.A.). 5,7-dibromo-N-(p-hydroxymethyl benzyl) isatin (isatin) was kindly provided by Dr Kara Perrow (University of Wollongong, Wollongong, Australia). 1-(N, 0-bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62), phorbol 12-myristate 13-acetate (PMA), adenosine-5'-O-(3-thio) triphosphate (ATP-3-S) and 7-amino-actinomycin-D (7AAD) were from Alexis Biochemicals (Lausen, Switzerland). A-438079 and AZ10606120 were from Tocris Bioscience (Park Ellisville, Missouri, U.S.A.). Human AB+ serum
was from the Australian Red Cross Blood Service (Penrith, Australia). Precision Plus Protein Standards (Dual colour), nitrocellulose membrane and Precision Protein StrepTactin-HRP conjugate were from Bio-Rad (Hercules, California, U.S.A.) and 4-20% gradient iGels were obtained from NuSep (Austell, Georgia, U.S.A.). Mini-Complete protease inhibitor cocktail tablets and interferon-gamma (IFN-γ) were from Roche Applied Science (Mannheim, Germany). Transforming growth factor (TGF)-β was from R&D Systems (Minneapolis, Minnesota, U.S.A.). SuperSignal® West Pico Chemiluminescent Substrate was from Pierce (Rockford, Illinois, U.S.A.). BD Falcon 70 µm cell strainers were from Becton Dickinson Labware (Franklin Lakes, New Jersey, U.S.A.) and used to filter adherent cell lines before flow cytometric analysis.

Murine anti-human P2X7 (clone L4) monoclonal antibody (mAb) (Buell et al., 1998) was purified and conjugated to fluorescein isothiocyanate (FITC) or Alexa Fluor 647 by Dr Ben Gu (University of Sydney, Penrith, Australia) or Dr Ronald Sluyter (University of Wollongong) respectively. Murine FITC- and Alexa 647-conjugated P2X7 isotype control mAb were from eBioscience (San Diego, California, U.S.A.). Rabbit anti-rat P2X7 polyclonal antibody (Ab) (C-terminal epitope) was from Alomone Labs (Jerusalem, Israel). Rabbit anti-actin Ab was from Sigma-Aldrich. FITC-conjugated murine anti-human CD14 (clone TUK4) and isotype control mAb were from Dako (Glostrup, Denmark). FITC-conjugated murine anti-human CD86 (clone 2331) and MHC class II (HLA-DR; clone L243) mAb were from BD Biosciences (San Jose, California, U.S.A.). HRP-conjugated goat anti-rabbit IgG Ab was from Rockland (Gillbertsville, Pennsylvania, U.S.A.).

2.2 Cell lines
The human leukemic monocytic cell line THP-1 was from the American Type Culture Collection (Rockville, Maryland, U.S.A.). The human myeloma cell line RPMI 8226, the epithelial colon cancer cell lines HCT-8 and Caco-2, and the acute myelogenous leukemic cell line KG-1 were obtained from the European Collection of Cell Cultures (Porton Down, UK). RPMI 8226, THP-1 and HCT-8 cells were maintained in RPMI-1640 medium supplemented with 10% FCS and 5 mM L-glutamine. Caco-2 and KG-1 cells were maintained in RPMI-1640 medium supplemented with 20% FCS, 5 mM L-glutamine and 100 µg/mL penicillin-streptomycin, according to the cell supplier’s recommendations. Cells were incubated at 37°C/5% CO₂ and passaged every 3-4 days as required. The adherent cell lines, HCT-8 and Caco-2, were harvested using 0.25% trypsin solution.

THP-1 cells were differentiated as described (Humphreys and Dubyak, 1998). Briefly, cells were plated at 1 × 10⁶ cells/mL and cultured for 3 days in 5 ml fresh complete media containing 1000 U/mL IFN-γ and 1 µg/mL LPS, with or without 5ng/mL TGF-β1. Untreated THP-1 cells were plated at 3 × 10⁵ cells/mL and cultured as above.

In some experiments HCT-8 cells were incubated with IFN-γ as described (Welter-Stahl et al., 2009). HCT-8 cells were plated at 1 × 10⁶ cells/mL and cultured for 3 days in 5 mL fresh complete media in the presence and absence of IFN-γ (0, 1, 10, 100 or 1000 U/mL).

2.3 Expression of P2X7 and other cell surface markers by immunofluorescence staining

Cells were washed twice with NaCl medium (145 mM NaCl, 5 mM KCl, 5 mM D-glucose, 0.1% BSA, 10 mM HEPES, pH 7.5). Cells (5 × 10⁵) were then incubated
for 20 min at room temperature with either specific or corresponding isotype control mAb in 100 µL NaCl medium containing 10% human AB+ serum, 0.01% NaN3 and 0.1 µg/mL 7AAD. The cells were washed once in 2 mL NaCl medium, centrifuged at 300 g for 5 min and the data was acquired using a LSR II flow cytometer and FACSDiva software (BD Biosciences). The mean fluorescence intensity (MFI) was determined using FlowJo software (Tree Star, Ashland, Oregon, U.S.A.).

2.4 P2X7 expression by immunoblotting

Whole cell lysates were prepared as described (Gu et al., 2004). Cells were washed three times at 300 g for 5 min in ice-cold PBS and then lysed (1 × 10⁷ cells/mL) in lysis buffer (50 mM Bis-Tris, 750 mM 6-aminohexanoic acid, 1 mM phenylmethylsulphonyl fluoride, 1% n-dodecyl β-D-maltoside and Mini-Complete protease inhibitors, pH 7.0) over 60 min, sheared ten times through a 21 gauge needle and centrifuged at 16,000 g for 10 min at 4ºC. Cell supernatants were then separated under reducing conditions (5% 2-mercaptoethanol) using 4-20% gradient iGels (50V for 120 min at 4ºC) and transferred to nitrocellulose membranes (Bio-Rad) (80V for 90 min at 4ºC). Nitrocellulose membranes were blocked overnight at 4ºC with Tris-buffered saline (250 mM NaCl, 50 mM Tris, pH 7.5) containing 0.2% Tween-20 and 5% skim milk powder, and then incubated for 2 h at room temperature with anti-P2X7 Ab diluted in Tris-buffered saline containing 0.2% Tween-20 and 5% skim milk powder. Membranes were washed three times over 30 min with Tris-buffered saline containing 0.2% Tween-20 and then incubated for 1 h at room temperature with HRP-conjugated anti-rabbit IgG Ab and Precision Protein StrepTactin-HRP conjugate and diluted in Tris-buffered saline containing 0.2% Tween-20 and 5% skim milk powder. Membranes
were washed as above, incubated with chemiluminescent substrate and visualised using Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.5 P2X7 expression by RT-PCR

Cells were washed once in sterile PBS medium. Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA was converted to cDNA using Superscript III One-Step RT-PCR System Platinum Taq DNA polymerase at 55°C for 30 min according to the manufacturer’s instructions. Previously described primers to P2X7 (forward primer 5′-GGATGGTGAACCAGCAGCTA-3′ reverse primer 5′-AAGCCACTGTACTGCCTTC-3′ (Skarratt et al., 2005) and β-actin (forward 5′-TGGTGGGCAATGGTCAGAAG-3′; reverse 5′-GTCCCGGCCAGCCAGGTCCAG-3′ (Wareham et al., 2009) were obtained from Sigma-Genosys (Castle Hill, Australia). PCR amplification was performed using 30 cycles (95°C, 15 s; 50°C, 30 s; 72°C, 60 s) for P2X7 and 30 cycles (95°C, 15 s; 61°C, 30 s; 72°C, 60 s) for β-actin. PCR products were separated on a 2% agarose gel and visualised using ethidium bromide staining.

2.6 Measurement of dye uptake by flow cytometry

P2X7 agonist-induced dye uptake into cells was measured using a fixed-time assay (Georgiou et al., 2005). Cells (5 × 10^5) resuspended in 1 mL NaCl medium (Section 2.3), KCl medium (150 mM KCl, 10 mM D-glucose, 0.1% BSA, 10 mM HEPES, pH 7.5) or sucrose medium (280 mM Sucrose, 5 mM KCl, 0.5 mM CaCl_2, 10 mM NMDG, 20 mM HEPES, 5 mM D-glucose, 0.1% BSA pH 7.4) or sucrose medium without Ca^{2+} were incubated with 25 μM ethidium^+ or 1 μM YO-PRO-1(Invitrogen, Eugene, OR, U.S.A.) in the absence or presence of nucleotide at 37°C, as indicated. In some experiments, cells were pre-incubated with 1 εM KN-62, 0.1 εM AZ10606120 or...
DMSO (diluent control), or in the presence or absence of 10 μM A-438079 or H2O (diluent control) for 15 min at 37°C before the addition of a nucleotide. Incubations were stopped by the addition of an equal volume of ice-cold MgCl2 medium (20 mM MgCl2, 145 mM NaCl, 5 mM KCl and 10 mM HEPES, pH 7.5) and centrifugation at 300 g for 5 min. Cells were then washed with 2 mL NaCl medium and analysed by flow cytometry. The MFI of dye uptake was determined using the FlowJo software.

2.7 IL-1β release measurements

ATP- and nigericin-induced IL-1β release from THP-1 cells was measured using a modification of a protocol used previously for human monocytes (Sluyter et al., 2004). THP-1 cells were suspended at 5 × 10^5 viable (trypan blue exclusion) cells/mL in RPMI-1640 medium containing 0.1% BSA (pH 7.5) and plated into 24-well plates (0.5 mL/well). Cells were incubated in the absence or presence of 3 mM ATP or 20 μM nigericin for 15 min at 37 °C/5% CO2. The samples were then centrifuged at 11,000 g for 10 s and the cell-free supernatants stored at -20°C for less than two weeks before assaying. Amounts of IL-1β were measured using a human IL-1β ELISA (eBioscience) according to the manufacturer's instructions.

2.8 Measurement of cell number by a colorimetric tetrazolium-based assay

Quantification of cell numbers following sustained application of P2X7 agonists was studied using the MTT colorimetric assay as described by Coutinho-Silva et al. (2005). Cells in their respective culture medium (3 × 10^3 cells/100 μL/well) were seeded in triplicate into 96 well plates and incubated for 24 hours at 37°C/5% CO2. Cells were then incubated in the absence or presence of nucleotide or isatin for a further 24 or 48 h (as indicated) at 37°C/5% CO2. In some experiments, cells were pre-incubated with 1
μM KN-62, 0.1 μM AZ10606120 or DMSO (diluent control), or in the presence of 10 μM A-438079 or H2O (diluent control) for 15 min at 37°C/5% CO2 before the addition of a nucleotide. The cells were then incubated with 0.45 mg/mL MTT solution during the final four hours of the 24 and 48 h incubations, after which 100 μL solubilisation solution (10% SDS in 0.01 M HCl) was added and the plates incubated overnight at 37°C. In some experiments, in the final four hours of the 24 and 48 h incubations, the cells were suspended in fresh culture media followed by the addition of 0.45 mg/mL MTT solution and solubilised thereafter. The mean absorbance was measured using a SpectaMax Plus384 microplate reader (Molecular Devices, Sunnyvale, California, U.S.A.) at a wavelength of 550 nm and a reference wavelength of 690 nm.

2.9 Measurement of cell death by trypan blue exclusion

Cells (1.5 × 10⁴ cells/0.5 mL/well) in their respective culture medium were seeded in triplicate in 24 well plates and incubated for 24 hours at 37°C/5% CO₂. Cells were then incubated in the absence or presence of nucleotide or 3 μM isatin for 24 or 48 h (as indicated) at 37°C/5% CO₂. At the end of 24 and 48 h incubations, aliquots of cells were mixed with an equal volume of 0.4% trypan blue solution in PBS. The total numbers of live and dead cells were then counted using a Bright-Line Haemocytometer (Hausser Scientific, Hersham, Pennsylvania, U.S.A.) according to manufacturer's instructions.

2.10 Morphological analysis of cell death

Cells 1.5 × 10⁴ cells/0.5 mL/well) in their respective culture medium were seeded in triplicate in 24-well plates and incubated for 24 hours at 37°C/5% CO₂. Cells were then incubated in the absence or presence of nucleotide or isatin for 24 or 48 h (as indicated) at 37°C/5% CO₂. At the end of 24 and 48 h incubations, a Leica (Mannheim,
Germany) DC 500 phase contrast microscope was used to analyse the cells. Images were captured using Leica Firecam software (Version 1.7.1). Apoptotic cells were identified according to classified morphological features of apoptotic cells including blebbing of the membrane, condensation of the nuclei and rounding up of cells (Kroemer et al., 2005; 2009).

2.11 Presentation of data, statistics and nomenclature

Data is presented as the mean ± standard deviation (SD). Differences between treatments were compared using either the unpaired Student's t-test for single comparisons to control samples or ANOVA for multiple comparisons (using Tukey's post test) using Prism 5 for PC version (GraphPad Software, San Diego, California, U.S.A.) with $P < 0.05$ considered significant. The nomenclature for P2X7, rather than P2X7, has been adopted according to recent recommendations (Jarvis and Khakh, 2009; Collingridge et al., 2009; Harmar et al., 2009).
Chapter 3: TGF-β1 prevents up-regulation of the P2X7 receptor in IFN-γ and LPS-treated myeloid leukemic THP-1 monocytic cells

3.1 Results

3.1.1 TGF-β1 prevents the up-regulation of P2X7 expression by IFN-γ and LPS

Differentiation of THP-1 cells with interferon-gamma (IFN-γ) and lipopolysaccharide (LPS) over three days up-regulates P2X7 expression and function (Humphreys and Dubyak, 1998). Previous work in our laboratory demonstrated that co-incubation with transforming growth factor-β1 (TGF-β1) abrogates cell-surface P2X7 expression and function in IFN-γ and LPS-differentiated THP-1 cells, with a maximal inhibitory response of 70% at 5 ng/mL and with an IC₅₀ of approximately 0.4 ng/mL (Tran, 2007; Georgiou and Sluyter, personal communication). Therefore to confirm these results, THP-1 cells were incubated in the presence of IFN-γ (1000U/mL) and LPS (1µg/mL) over 72 hours as previously described (Humphreys and Dubyak, 1998), in the absence or presence of TGF-β1 (10 ng/mL) and the amount of cell-surface P2X7 was measured by immunolabelling with an anti-P2X7 monoclonal antibody (mAb) and flow cytometry. Cell-surface P2X7 was also examined in THP-1 cells incubated in the absence of cytokines and LPS (undifferentiated THP-1 cells). Undifferentiated cells expressed negligible amounts of cell-surface P2X7, whereas cell-surface P2X7 was significantly upregulated following differentiation with IFN-γ and LPS (mean fluorescence intensity, MFI of 1.8 ± 0.5 and 28.8 ± 9.6 respectively, P < 0.01; n = 5, Fig. 3.1 A,B). Consistent with Tran (2007) and Georgiou and Sluyter (personal communication) co-incubation of THP-1 cells with IFN-γ, LPS and TGF-β1 significantly abrogated cell-surface P2X7 expression (MFI of 10.82 ± 4.2, P < 0.01; n = 5, Fig. 3.1C) compared to THP-1 cells incubated in the presence of IFN-γ and LPS.
Fig. 3.1 TGF-β1 prevents the up-regulation of P2X7 expression by IFN-γ and LPS in THP-1 monocytes. THP-1 cells were incubated for 3 days in the (A) absence or (B, C) presence of 1000 U/mL IFN-γ and 1 μg/mL LPS, and in the (B) absence or (C) presence of 10 ng/ml TGF-β1. Cells were labelled with FITC-conjugated anti-P2X7 (solid line) or isotype control (shaded) mAb and 7-amino-actinomycin (7AA)-D, and the relative cell-surface expression (MFI) was determined by flow cytometry. Representative results from five experiments are shown.

3.1.2 TGF-β1 prevents the up-regulation of P2X7 function by IFN-γ and LPS

Next, to confirm that co-incubation of IFN-γ and LPS-differentiated THP-1 cells with TGF-β1 can abrogate P2X7 function, THP-1 cells were cultured as above and the ability of ATP and BzATP to induce ethidium⁺ uptake was measured by flow cytometry. Differences in the amount of cell-surface P2X7 (Section 3.1.1) corresponded with differences in ATP-BzATP-induced ethidium⁺ uptake. Both ATP and BzATP failed to induce ethidium⁺ uptake into undifferentiated THP-1 cells with values similar to basal ethidium⁺ uptake (Fig. 3.2). As expected ATP induced significant ethidium⁺ uptake into differentiated THP-1 cells compared to basal ethidium⁺ uptake (Fig. 3.2). Moreover, the most potent P2X7 agonist, BzATP, (North, 2002), induced significantly higher levels of

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ethidium\(^+\) uptake into differentiated THP-1 cells compared to basal and ATP-induced ethidium\(^+\) uptake levels (Fig. 3.2). Both ATP- and BzATP-induced ethidium\(^+\) uptake were significantly lower in IFN-\(\gamma\) and LPS-treated THP-1 cells co-incubated with TGF-\(\beta1\) compared to IFN-\(\gamma\) and LPS-treated THP-1 cells incubated in the absence of TGF-\(\beta1\). (Fig. 3.2)

![Graph showing relative ethidium\(^+\) uptake](image)

**Fig. 3.2** TGF-\(\beta1\) prevents the up-regulation of P2X7 function by IFN-\(\gamma\) and LPS in THP-1 monocytes. THP-1 cells were incubated for 3 days in the presence of 1000 U/mL IFN-\(\gamma\) and 1 \(\mu\)g/mL LPS in the absence or presence of 10 ng/mL TGF-\(\beta1\), or in the absence of IFN-\(\gamma\) and LPS. Cells were resuspended in NaCl medium at 37\(^\circ\)C, ethidium\(^+\) (25 \(\mu\)M) was added, followed 30 s later by the addition of 1 mM ATP or 200 \(\mu\)M BzATP. After 5 min, incubations were stopped by addition of MgCl\(_2\) medium and centrifugation, and the ethidium\(^+\) uptake (MFI) determined by flow cytometry. Results are shown as the mean ± SD (\(n = 3\)); **\(P < 0.01\) and ***\(P < 0.001\).

To demonstrate that the effect of TGF-\(\beta1\) on P2X7 function was not limited to ethidium\(^+\) uptake, ATP-induced YO-PRO-1\(^{2+}\) uptake in THP-1 cells, resuspended in
NaCl medium was examined as described (Gudipaty et al., 2001). ATP induced significant YO-PRO-1^{2+} uptake into IFN-γ and LPS-treated THP-1 cells, but not into undifferentiated cells (Fig. 3.3). The presence of TGF-β1 during the incubation of THP-1 cells with IFN-γ and LPS significantly decreased ATP-induced YO-PRO-1^{2+} uptake (Fig. 3.3).

**Fig. 3.3** TGF-β1 prevents the up-regulation of ATP-induced YO-PRO-1^{2+} uptake in IFN-γ and LPS-treated THP-1 monocytes. THP-1 cells were incubated for 3 days with 1000 U/ml IFN-γ and 1 μg/ml LPS in the absence or presence of 10 ng/ml TGF-β1, or in the absence of IFN-γ and LPS. Cells were resuspended in NaCl medium containing 1 μM YO-PRO-1^{2+}, and incubated in the absence or presence of 1 mM ATP at 37°C for 15 min. Incubations were stopped by addition of NaCl medium containing 20 mM MgCl₂ medium and centrifugation, and the YO-PRO-1^{2+} uptake (MFI) determined by flow cytometry. Results are shown as mean ± SD (n = 3); ***P < 0.001.

Next, ATP-induced interlukin (IL)-1β release from THP-1 cells was examined as described (Sluyter et al., 2004). Nigericin, which induces IL-1β release independently
of P2X7 activation, was used as a positive control. ATP induced a small but significant release of IL-1β from IFN-γ and LPS-treated THP-1 cells, but not undifferentiated cells (Fig. 3.4). The presence of TGF-β1 during the incubation of THP-1 cells with IFN-γ and LPS decreased ATP-induced IL-1β release by 79 ± 26%, although this failed to reach statistical significance (Fig. 3.4). This decrease in ATP-induced IL-1β release was not due to differences in IL-1β synthesis or release mechanisms between the two groups as nigericin caused a similar IL-1β release from IFN-γ and LPS-treated cells incubated in the absence or presence of TGF-β1 (Fig. 3.4).

**Fig. 3.4** TGF-β1 prevents the up-regulation of ATP-induced IL-1β release in IFN-γ and LPS-treated THP-1 monocytes. THP-1 cells were incubated for 3 days with 1000 U/mL IFN-γ and 1 μg/mL LPS in the absence or presence of 5 ng/mL TGF-β1, or in the absence of IFN-γ and LPS. Cells were suspended in RPMI-1640 medium containing 0.1% BSA, and incubated in the absence or presence of 3 mM ATP or 20 μM nigericin for 15 min at 37°C/5% CO₂. The samples were centrifuged and the amount of IL-1β in cell-free supernatants measured by ELISA. Results are shown as mean ± SD (n = 3); *P < 0.05 and **P < 0.01.
3.1.3 TGF-β1 prevents the up-regulation of total P2X7 by IFN-γ and LPS

Human monocytes contain a large pool of intracellular P2X7 (Gu et al., 2000), which traffics to the cell surface during differentiation to macrophages (Gudipaty et al., 2001). Therefore, to determine if the effect of TGF-β1 in differentiated THP-1 cells is due to altered P2X7 trafficking or to a decrease in total P2X7 protein, whole lysates of THP-1 cells were prepared and the amount of P2X7 was examined by immunoblotting. Actin was included as a loading control. Immunoblotting with an anti-P2X7 polyclonal antibody (Ab) failed to detect any bands in untreated THP-1 cells despite the presence of actin (Fig. 3.5). In contrast, this procedure revealed the presence of a major band at 75 kDa, the predicted size of glycosylated P2X7, in IFN-γ and LPS-treated THP-1 cells incubated in the presence or absence of TGF-β1 (Fig. 3.5). A minor band at 55 kDa was also detected in these cells (Fig. 3.5). Of note, the intensity of the 75 and 55 kDa bands were consistently reduced in the IFN-γ and LPS-treated THP-1 cells incubated in the presence of TGF-β1 compared to IFN-γ and LPS-treated cells incubated in the absence of TGF-β1, despite the presence of equal amounts of actin (Fig. 3.5).
**Fig. 3.5** TGF-β1 prevents the up-regulation of total P2X7 in IFN-γ and LPS-treated THP-1 monocytes. Whole lysates of untreated THP-1 cells, as well as of THP-1 cells incubated for 3 days with 1000 U/mL IFN-γ and 1 μg/mL LPS in the absence or presence of 10 ng/mL TGF-β1 were separated by electrophoresis, transferred to nitrocellulose membrane and probed with either (**Left panel**) anti-P2X7 Ab or (**right panel**) anti-actin Ab. Molecular weight markers, detected using StrepTactin-HRP, are shown in the far left lanes of each blot. Results are representative of three experiments.

### 3.1.4 TGF-β1 prevents the up-regulation of P2X7 mRNA by IFN-γ and LPS

Incubation of THP-1 cells with IFN-γ and LPS up-regulates P2X7 mRNA (Humphreys and Dubyak, 1998). Therefore, to determine if the effect of TGF-β1 in differentiated THP-1 cells is due to a decrease in P2X7 mRNA, RNA was isolated from THP-1 cells and the amount of P2X7 mRNA examined by RT-PCR. ß-actin was included as a house keeping control and RPMI 8226 myeloma cells were used as positive control, which express high amounts of functional P2X7 (Farell, 2008). P2X7 mRNA was present in undifferentiated THP-1 cells, and incubation with IFN-γ and LPS up-regulated P2X7 mRNA expression (Fig. 3.6). In contrast, P2X7 mRNA expression in IFN-γ and LPS-treated cells incubated in the presence of TGF-β1 was lower than IFN-γ and LPS-treated cells incubated in the absence of TGF-β1, despite similar expression of ß-actin mRNA (Fig. 3.6).
**Fig. 3.6** TGF-β1 prevents the up-regulation of P2X7 mRNA in IFN-γ and LPS-treated THP-1 monocytes. *Left panel* P2X7 and *right panel* β-actin mRNA from untreated THP-1 cells and RPMI 8226 cells, as well as from THP-1 cells incubated for 3 days with 1000 U/mL IFN-γ and 1 μg/mL LPS in the absence or presence of 10 ng/mL TGF-β1 were amplified by RT-PCR, the products separated on a 2% agarose gel and visualised using ethidium bromide staining. A negative control for P2X7 primers was set up by substituting RNA with distilled water. Results are representative of three experiments.

3.1.5 TGF-β1 prevents the up-regulation of CD86 but not CD14 or MHC class II by IFN-γ and LPS

Tamai *et al* (2003) have shown that co-stimulation of undifferentiated THP-1 cells with IFN-γ and LPS markedly enhances cell surface CD14 expression. Therefore, it was determined if the TGF-β1 effect on THP-1 cells incubated with IFN-γ and LPS was specific to P2X7 or could also affect other cell-surface receptors. THP-1 cells incubated with IFN-γ and LPS in the presence or absence of TGF-β1, as well as
undifferentiated cells were labelled with a panel of mAb and the expression of cell-surface markers examined by flow cytometry. Undifferentiated THP-1 cells expressed negligible to low levels of cell-surface CD14, CD86 and MHC class II, as well as P2X7 (Fig. 3.7). Differentiation of THP-1 cells with IFN-γ and LPS increased the cell-surface expression of P2X7, CD14, CD86 and MHC class II (Fig. 3.7). As above (Fig. 3.1), the presence of TGF-β1 during the incubation of THP-1 cells with IFN-γ and LPS significantly decreased P2X7 expression (Fig. 3.7). Similarly, TGF-β1 significantly decreased CD86 expression on cells incubated with IFN-γ and LPS (Fig. 3.7). In contrast, CD14 and MHC class II expression was similar on IFN-γ and LPS-treated THP-1 cells incubated in the presence or absence of TGF-β1 (Fig. 3.7).
**Fig. 3.7** TGF-β1 prevents the up-regulation of CD86 but not CD14 or MHC class II in IFN-γ and LPS-treated THP-1 monocytes. Untreated THP-1 cells, as well as THP-1 cells incubated for 3 days with 1000 U/mL IFN-γ and 1 μg/mL LPS in the absence or presence of 10 ng/mL TGF-β1, were labelled with fluorochrome-conjugated mAb and 7AAD, and the relative cell-surface expression (MFI) determined by flow cytometry. Results are expressed as the mean ± SD (n = 5); *P < 0.05, **P < 0.01 and ***P < 0.001.
3.2 Discussion

P2X7 expression and function can be up-regulated in the human leukemic monocytic cell line THP-1 upon incubation with IFN-γ and LPS (Humphreys and Dubyak, 1998 and Humphreys et al., 1998). Using this model of monocyte/macrophage differentiation, Tran (2007) and Georgiou and Sluyter (personal communication) demonstrated that TGF-β1 prevents the up-regulation of P2X7 expression and function (ATP-induced ethidium+ uptake) by IFN-γ and LPS; however the mechanism has never been elucidated. Results in this chapter confirm that TGF-β1 prevents the up-regulation of P2X7 in IFN-γ and LPS-treated THP-1 cells. Moreover, results in this chapter indicated this effect of TGF-β1 was not limited to ethidium+ uptake, as ATP-induced YO-PRO-12+ uptake and IL-1β release was also impaired in IFN-γ and LPS-treated THP-1 cells incubated with TGF-β1 compared to IFN-γ and LPS-treated cells incubated without TGF-β1. Although, it should be noted that the amount of ATP-induced IL-1β released from THP-1 cells incubated for 3 days with IFN-γ and LPS is lower than the amount of ATP-induced IL-1β release from THP-1 cells incubated for 3 h with PMA and then for 6-24 h with LPS (Grahames et al., 1999; MacKenzie et al., 2001) or for 2 days with IFN-γ and then for 4 h with LPS (Gudipaty et al., 2003). The reason for this difference is unknown, but prolonged (3 day) incubation of THP-1 cells with LPS and/or possibly IFN-γ may result in the loss of IL-1β prior to ATP or nigericin incubation. Alternatively, 3-day incubation of THP-1 cells with IFN-γ and LPS may not induce large amounts IL-1β synthesis.

The mechanism by which TGF-β1 abrogates the up-regulation of cell-surface P2X7 by IFN-γ and LPS in THP-1 cells has been elucidated. Immunoblotting of whole cell lysates with anti-P2X7 Ab demonstrated that TGF-β1 prevents the synthesis of the
total amount of P2X7 protein in differentiated THP-1 cells. Moreover, this reduction in P2X7 protein paralleled a decrease in P2X7 mRNA expression suggesting that transcription rather than translation is abrogated by TGF-β1 in THP-1 cells incubated with IFN-γ and LPS. However, we cannot exclude the possibility that TGF-β1 may be involved in some post-transcription event such as increased P2X7 mRNA decay, as TGF-β1 can increase the rate of decay of chemokine mRNA induced by LPS in murine macrophages (Dai et al., 2003). It appears unlikely that the inhibitory effect of TGF-β1 is due to impaired trafficking of P2X7 as total P2X7 protein amounts were reduced following TGF-β1 treatment, although impaired trafficking cannot be fully excluded as an additional, albeit minor, mechanism. The inhibitory effect of TGF-β1 is not due to increased amounts of a P2X7 variant, which impairs P2X7 expression and function as observed in some epithelial cell lines (Gordeski, 2009), as immunoblotting revealed a decrease in both full length P2X7 (75 kDa) and a shorter P2X7 variant (55 kDa). The reduced P2X7 function following TGF-β1 incubation was also not due to increased levels of the ecto-ATPDase CD39 (Tran, 2007). THP-1 cells, regardless of treatment, failed to express cell-surface CD39; however the possibility that other ectonucleotidases (Yegutkin, 2008) play an ancillary role in the reduced P2X7 function in TGF-β1-treated THP-1 cells cannot be excluded.

The THP-1 cell line is a well-established model used to study the P2X7 receptor and its downstream effects. Data in this chapter confirms the presence of functional P2X7 in THP-1 cells following differentiation with IFN-γ and LPS, as originally observed by others (Humphreys and Dubyak, 1998; Humphreys et al., 1998). Data in this chapter also confirms the absence of functional P2X7 in undifferentiated THP-1 cells as observed by others (Humphreys and Dubyak, 1996; 1998). In contrast another
group (Aga et al., 2002) has demonstrated the presence of functional P2X7 in undifferentiated THP-1 cells whereby the most potent P2X7 agonist, BzATP induced mitogen activated protein kinase activation, NF-κB translocation and IL-1β release. The reasons for these differences are unknown but may reflect differences in cell lines between laboratories, differences in the relative sensitivities of the different assays, or differences in the relative activation thresholds including amounts of cell-surface P2X7 expression required for different P2X7-mediated events. However it should be noted that BzATP failed to induce significant cation dye uptake and IL-1β release in undifferentiated THP-1s in my study. The detection of P2X7 mRNA and low to negligible amounts of cell-surface P2X7, in undifferentiated THP-1 cells in this chapter suggests these cells may contain low amounts of functional P2X7 but which were undetectable by the cation uptake and IL-1β release assays used.

The permeability pathways that mediate the passage of large organic ions following P2X7 activation have not been fully elucidated, although recent data indicates that the dissociation of non-muscle myosin from the P2X7 complex may be involved in P2X7 pore formation (Gu et al., 2009). In this chapter, ATP induced the uptake of ethidium\(^+\) (314 Da) and YO-PRO-1\(^{2+}\) (375 Da), while previous data from our laboratory demonstrated that ATP induced ethidium\(^+\) but not propidium\(^{2+}\) (415 Da) into differentiated THP-1 cells (Sluyter, personal communication). These results are similar to those observed in human lymphocytes (Wiley et al, 1993) and dendritic cells (Sluyter and Wiley, 2002), but contrasts ATP-induced uptake of propidium\(^{2+}\) uptake in osteoblasts (Panupinthu et al., 2008) and P2X7-transfected HEK-293 cells (Milius et al., 2007). The opening of pannexin-1 has also been implicated in the P2X7-mediated uptake of ethidium\(^+\) in macrophages and P2X7-transfected HEK-293 cells (Pelegrin and
Suprenant, 2006), but the presence of pannexin-1 in THP-1 cells and whether this hemichannel is permeable to propidium$^{2+}$ is unknown. Nevertheless our data suggests that propidium$^{2+}$ may enter via a different permeability pathway to that of ethidium$^+$, or that the pore size of the P2X7 permeability pathway may differ between cell types.

In addition to P2X7, IFN-$\gamma$ and LPS up-regulated the expression of CD14, CD86 and MHC class II in THP-1 cells. Although various inflammatory stimuli can up-regulate each of these receptors in THP-1 cells (Tamai et al., 2003; Miyazawa et al., 2007; Tomoda et al., 1992), in relation to incubation with both IFN-$\gamma$ and LPS this has only been previously demonstrated for CD14 (Tamai et al., 2003). Thus, data in this chapter demonstrates that in addition to CD14, incubation with both IFN-$\gamma$ and LPS, can also up-regulate CD86 and MHC class II in THP-1 cells. It should be noted however, that in two of the five experiments the up-regulation of MHC class II was negligible despite up-regulation of P2X7, CD14 and CD86. Sub-analysis of the three remaining experiments showed that incubation with IFN-$\gamma$ and LPS significantly up-regulated MHC class II in THP-1 cells, and that co-incubation with TGF-$\beta$I did not abrogate expression of this cell-surface molecule (results not shown). The null effect of TGF-$\beta$I on CD14 and MHC class II in THP-1 monocytes in the presence of both IFN-$\gamma$ and LPS contrasts that observed with macrophages treated with either IFN-$\gamma$ or LPS alone, where TGF-$\beta$I inhibits the expression of either CD14 or MHC class II on IFN-$\gamma$- or LPS-treated macrophages respectively (Imai et al., 2000; Nandan and Reiner, 1997). The reasons for these differing results are unknown. The action of TGF-$\beta$I however varies depending on the combination of cytokines within the extracellular environment (Dennler, Goumans and Dijke, 2005) thus providing a possible explanation for the differences observed between the various studies, apart from differences in cell type.
The inhibitory effect of TGF-β1 on P2X7 in IFN-γ and LPS-treated THP-1 cells suggests that TGF-β1 may impair the expression and function of P2X7 on macrophages during inflammatory and immune responses. P2X7 activation can stimulate the NALP3 inflammasome to cause the subsequent release of IL-1β from macrophages (Ferrari et al., 2006). Thus, TGF-β1 released during or after infection may serve to reduce ATP-induced IL-1β release by down-regulating P2X7 in macrophages to limit tissue injury or to help resolve the immune response. In contrast, the absence of TGF-β1 may allow maximal P2X7 expression and function to promote ATP-induced IL-1β release during the immune response. Our findings (Tran, 2007; Georgiou and Sluyter, personal communication; this chapter) also suggest that aberrant TGF-β1 production may contribute to immune-related disorders in which P2X7 is involved. For example, the absence of TGF-β1 may help to maintain ATP-induced IL-1β release in rheumatoid arthritis where a role for P2X7 has been established (Labasi et al., 2002). Alternatively, increased TGF-β1 release during infection may down-modulate P2X7 expression in macrophages resulting in impaired ATP-induced killing of intracellular pathogens. In this regard, loss of P2X7 function, resulting from polymorphic variations in the P2RX7 gene, results in impaired ATP-induced killing of intracellular Mycobacterium tuberculosis and Toxoplasma gondii, and increased susceptibility to tuberculosis and toxoplasmosis respectively (Fernando et al., 2007; Lees et al., 2010) highlighting the requirement for optimal P2X7 expression and function to control these pathogens. Moreover, both these pathogens can induce TGF-β1 release to suppress macrophage activation to allow their survival and to promote disease (Li et al., 2006), suggesting that down-regulation of P2X7 by this cytokine during infection may provide an additional mechanism by which these pathogens can evade destruction. Finally, TGF-β1
may also modulate P2X7 expression and function within the tumour microenvironment. TGF-β1 can promote and suppress tumour growth by acting on malignant, stromal and immune cells. Thus it is possible that TGF-β1 may act on any of these cell types expressing P2X7 to modify the progression of malignant diseases. It will be of future importance to determine if TGF-β1 can prevent the up-regulation of P2X7 on macrophages differentiated from primary monocytes, as well as the effect of TGF-β1 on P2X7 in other normal and malignant cell types.

In conclusion, this study confirms that TGF-β1 can prevent the up-regulation of P2X7 expression and function by IFN-γ and LPS in THP-1 monocytes, and indicates that TGF-β1 prevents P2X7 upregulation by preventing the upregulation of P2X7 mRNA and the subsequent synthesis of P2X7. This suggests that TGF-β1 may limit P2X7-mediated processes during inflammatory and immune responses or P2X7 function in malignant cells.
Chapter 4: Colon epithelial carcinoma HCT-8 and Caco-2 cells lack functional P2X7 receptors

4.1 Results

4.1.1 HCT-8 and Caco-2 cells express low levels of P2X7

P2X7 is present in normal and malignant primary epithelial cells of the intestine (Groschel et al., 1999) and skin (Greig et al., 2003; Georgiou et al., 2005). In addition, Coutinho-Silva et al (2005) have demonstrated that ATP and BzATP induce death of the human colon epithelial carcinoma cell lines, HCT-8 and Caco-2 in a manner characteristic of P2X7, but direct evidence for P2X7 in this process was lacking. Therefore, to study the presence of P2X7 on these cell lines, HCT-8 and Caco-2 cells were labelled with anti-P2X7 monoclonal antibody (mAb) and examined by flow cytometry. Human multiple myeloma RPMI 8226 cells, which express high amounts of functional P2X7 (Farell, 2008), were used as a positive control. The RPMI 8226 cells were found to express high levels of P2X7 on the cell-surface compared to the isotype control (mean fluorescence intensity, MFI of 34.9 ± 4.7 and 5.7 ± 2.8 respectively, $P < 0.01; n = 3$, Fig. 4.1). In contrast, low levels of cell surface P2X7 were detected on HCT-8 and Caco-2 cells relative to the isotype control (HCT-8, MFI of 9.2 ± 3.4 and 5.7 ± 1.1 respectively; Caco-2, 13.1 ± 3.9 and 10.3 ± 6.0 respectively; $n = 3$, Fig. 4.1B, C).
Fig. 4.1 HCT-8 and Caco-2 cells express low levels of cell surface P2X7. (A) RPMI 8226 (positive control), (B) HCT-8 and (C) Caco-2 cells were labelled with FITC-conjugated anti-P2X7 (solid line) or isotype control (shaded) mAb and 7-amino-actinomycin-D (7AAD), and the relative cell surface expression (MFI) determined by flow cytometry. Representative results from three experiments are shown.

To determine whether this low amount of P2X7 on HCT-8 and Caco-2 cells was due to low cell surface P2X7 expression or non-specific binding of the anti-P2X7 mAb, immunoblotting was performed on whole cell lysates of RPMI 8226, HCT-8 and Caco-2 cells. Immunoblotting of RPMI 8226 cells with an anti-P2X7 polyclonal antibody (Ab) revealed the presence of a major band at 75 kDa (Fig. 4.2), the predicted size of glycosylated P2X7. Intense bands were present at 68 and 42 kDa in RPMI 8226 cells (Fig. 4.2), which most likely correspond to non-glycosylated P2X7, and a P2X7 variant or breakdown product. Immunoblotting with the same P2X7 Ab also revealed the presence of strong 68 and 42 kDa bands in HCT-8 and Caco-2 cells (Fig. 4.2). In contrast, these cells expressed only a faint band at 75 kDa (Fig. 4.2).
Fig. 4.2 HCT-8 and Caco-2 cells express low amounts of full-length P2X7. Whole lysates of RPMI 8226, HCT-8 and Caco-2 cells were separated by electrophoresis, transferred to nitrocellulose membrane and probed with anti-P2X7 antibody. Molecular weight markers, detected using StrepTactin-HRP, are shown in the far left lane. Results representative of four experiments.

4.1.2 P2X7 agonists do not induce ethidium\(^+-\) uptake in to HCT-8 and Caco-2 cells.

The above results indicate that HCT-8 and Caco-2 cells express low amounts of P2X7. To determine if these cells express functional P2X7, the ability of ATP to induce ethidium\(^+-\) uptake over 5 min into HCT-8 and Caco-2 cells was measured previously as for THP-1 cells (Chapter 3). RPMI 8226 cells were used as a positive control. As predicted, ATP induced significant ethidium\(^+-\) uptake into RPMI 8226 myeloma cells at 5 min compared to basal levels (Fig. 4.3A). In contrast, 5 min incubation with ATP failed to induce ethidium\(^+-\) uptake into HCT-8 and Caco-2 cells compared to the basal levels (Fig. 4.3B,C).
**Fig. 4.3** ATP does not induce ethidium$^+$ uptake into HCT-8 and Caco-2 cells. (A) RPMI 8226, (B) HCT-8 and (C) Caco-2 cells in NaCl medium containing 25 µM ethidium$^+$ were incubated at 37°C for 5 min in the absence (basal) or presence of 1 mM ATP. Incubations were stopped by addition of MgCl$_2$ medium and centrifugation, and the ethidium$^+$ uptake (MFI) determined by flow cytometry. Results are shown as the mean ± SD ($n=3$); ***$P<0.001$.

It was possible, that 5 min incubation with ATP was insufficient to detect the functional activity of the low amounts of cell-surface P2X7 present on HCT-8 and Caco-2 cells. Therefore, cells were incubated in the presence and absence of ATP or the most potent P2X7 agonist, 2(3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP) over 30 min. Ethidium$^+$ uptake in the absence or presence of the P2X7 agonists, in both the cell lines was variable but tended to increase over time with the exception of basal ethidium$^+$ uptake in Caco-2 cells (Fig. 4.4). Nevertheless ATP and BzATP failed to induce significant ethidium$^+$ uptake compared to basal levels at corresponding time points in either cell type (Fig. 4.4).
Fig. 4.4 Prolonged incubation with P2X7 agonists do not induce ethidium$^+$ uptake into HCT-8 and Caco-2 cells. (A) HCT-8 and (B) Caco-2 cells in NaCl medium containing 25 µM ethidium$^+$ were incubated at 37°C for up to 30 min (as indicated) in the absence (basal) or presence of 1 mM ATP or 200 µM BzATP. Incubations were stopped by addition of MgCl$_2$ medium and centrifugation, and the ethidium$^+$ uptake (MFI) determined by flow cytometry. Results are shown as the mean ± SD (n = 3).

The maximal response and efflux rates of P2X7 to ATP and BzATP are higher in KCl medium compared to NaCl medium (Stevenson et al., 2009; Wiley et al., 1993). While, Michel et al. (1999) have previously shown that the potency of BzATP for human P2X7 is approximately 20-fold higher in sucrose medium compared to NaCl medium, similar to that used above (Fig. 4.2 and 4.3). Moreover, P2X7 activation can be detected in malignant cell lines resuspended in sucrose but not NaCl medium (Alqallaf et al., 2009; Zhang et al., 2007). Therefore, HCT-8 cells were suspended in KCl or sucrose medium and incubated in the presence and absence of ATP or BzATP, and the relative levels of ethidium$^+$ uptake over 5 min were measured. Cells in NaCl
medium were studied as a comparison. ATP and BzATP failed to induce ethidium\(^+\) uptake into HCT-8 cells in KCl and sucrose medium, as well as NaCl medium (Fig. 4.5).

**Fig. 4.5** P2X7 agonists do not induce ethidium\(^+\) uptake into HCT-8 cells in KCl or sucrose medium. HCT-8 cells in (A) NaCl, (B) KCl or (C) sucrose medium containing 25 \(\mu\)M ethidium\(^+\) were incubated at 37\(^\circ\)C for 5 min in the absence (basal) or presence of 1 mM ATP or 200 \(\mu\)M BzATP. Incubations were stopped by addition of MgCl\(_2\) medium and centrifugation and the ethidium\(^+\) uptake (MFI) was determined by flow cytometry. Results are shown as the mean ± SD \((n = 3)\).

Since, neither ATP nor BzATP could induce ethidium\(^+\) uptake into HCT-8 cells in KCl or sucrose medium over 5 min, the ability of ATP and BzATP to induce ethidium\(^+\) uptake into HCT-8 and Caco-2 cells over 30 min was examined. Both basal and nucleotide-induced ethidium\(^+\) uptake into both cell lines was variable and no regular trend was observed (Fig. 4.6). Moreover, both ATP and BzATP failed to induce significant ethidium\(^+\) uptake in HCT-8 and Caco-2 cells in sucrose medium over 30 min compared to basal levels (Fig. 4.6).
**Fig. 4.6** ATP and BzATP do not induce ethidium$^+$ uptake in HCT-8 and Caco-2 cells in sucrose medium over extended incubation. (A) HCT-8 and (B) Caco-2 cells in sucrose medium containing 25 µM ethidium$^+$ were incubated at 37°C for over 30 min in the absence (basal) or presence of 1 mM ATP or 200 µM BzATP. Incubations were stopped by addition of MgCl$_2$ medium and centrifugation, and the ethidium$^+$ uptake (MFI) was determined by flow cytometry. Results are shown as the mean ± SD ($n = 3$).

Activation of P2X7 is impaired in the presence of extracellular Ca$^{2+}$ (Virginio et al., 1997). Of note, the sucrose medium of Michel et al (1999) and as used above contains 0.5 mM CaCl$_2$. Therefore, ATP- and BzATP-induced ethidium$^+$ uptake was assessed in HCT-8 and Caco-2 cells resuspended in sucrose medium without Ca$^{2+}$. Again basal and nucleotide-induced ethidium$^+$ uptake was variable over three independent experiments and no regular trend was observed. Moreover, both ATP and BzATP failed to induce significant ethidium$^+$ uptake in HCT-8 and Caco-2 cells in sucrose medium over 30 min compared to basal levels (Fig. 4.7).
Fig. 4.7 ATP and BzATP do not induce ethidium$^+$ uptake in HCT-8 and Caco-2 cells in sucrose medium without Ca$^{2+}$. (A) HCT-8 and (B) Caco-2 cells in sucrose medium (without Ca$^{2+}$) containing 25 µM ethidium$^+$ were incubated at 37°C for over 30 min in the absence (basal) or presence of 1 mM ATP or 200 µM BzATP. Incubations were stopped by addition of MgCl$_2$ medium and centrifugation and the ethidium$^+$ uptake (MFI) was determined by flow cytometry. Results are shown as the mean ± SD ($n = 3$).

4.1.3. P2X7 agonists do not induce death of HCT-8 and Caco-2 cells

Using a MTT (tetrazolium-based colourimetric) cell viability assay, Coutinho-Silva et al (2005) demonstrated that ATP could induce death of HCT-8 and Caco-2 cells, although a direct role for P2X7 in this process was not established. Nevertheless, the possibility remained that P2X7 activation induces cell death but not ethidium$^+$ uptake in these cells, or that the MTT assay may be a more sensitive assay than ethidium$^+$ uptake in detecting functional P2X7. Therefore, ATP- and BzATP-induced death of HCT-8 and Caco-2 cells over 24 and 48 h incubation was assessed using the MTT assay as described (Coutinho-Silva et al., 2005). The MTT assay provides an indirect measure of cell death by measuring cell numbers (derived from
absorbance readings of formazan crystal formation converted from MTT by metabolically active cells). Incubation of HCT-8 and Caco-2 cells with either agonist for 24 and 48 h failed to induce cell death in to HCT-8 and Caco-2 cells (Fig. 4.8A,C). In one out of three experiments, BzATP induced low but insignificant levels of death in Caco-2 cells, at 48 h, compared to the basal (results not shown). The results however did not follow a consistent pattern; all three independent experiments gave variable results. Therefore, in an alternative more conventional protocol after incubation with nucleotide, the culture medium was not replaced, prior to the addition of MTT. Similar results however were observed. Both ATP and BzATP failed to induce significant cell death in HCT-8 and caco-2 cells at both the time points (Fig. 4.8B,D). Again the three set of results were found to be quite variable.
**Fig. 4.8** ATP and BzATP do not induce cell death in HCT-8 and Caco-2 cells. (A, B) HCT-8 cells and (C, D) Caco-2 cells in complete culture medium were incubated in the absence (basal) or presence of 1 mM ATP or 200 µM BzATP at 37°C/5% CO2 for 24 hrs and 48 hrs. (A,C) At the end of 24 and 48 hours, the culture media of individual wells was substituted with 100 µl of fresh culture medium before MTT addition. (B,D) At the end of 24 and 48 hours, MTT was added directly to the culture media without any substitution. (A-D) Cells were incubated with MTT for 4 h, before overnight incubation with solubilization solution at 37°C. Absorbances were then read at 550 and 690 nm. Results are mean absorbances (A_{550 nm}-A_{690 nm}) ± SD of triplicate wells; representative results from two experiments are shown.
4.1.4 IFN-γ does not upregulate P2X7 in HCT-8 cells

During the course of this thesis, Welter-Stahl et al (2009) showed that P2X7 can be upregulated in HCT-8 cells following incubation with an interferon-gamma (IFN-γ). Therefore, in an attempt to upregulate P2X7 expression and/or function in HCT-8 cells, cells were incubated in the presence of increasing concentrations of IFN-γ, as described (Welter-Stahl et al., 2009), and cell-surface P2X7 expression and BzATP-induced ethidium⁺ uptake measured. IFN-γ failed to upregulate either P2X7 expression or function in HCT-8 cells (Fig. 4.9A,B).

**Fig. 4.9** IFN-γ does not upregulate P2X7 expression and function in HCT-8 cells. HCT-8 cells were cultured for 3 days in 5 mL complete media in increasing concentrations of IFN-γ (as indicated). The cells were harvested and analysed for (A) cell-surface P2X7 expression (as described in Fig. 4.1) and (B) 200 µM BzATP-induced ethidium⁺ uptake (as described in Fig. 4.3). Relative P2X7 expression is shown as the difference in binding between P2X7 and isotype control. Results are shown as the mean ± SD (n = 3).
4.2 Discussion
The P2X7 receptor is suggested to play an important role in the regulation of epithelial cell growth (Gordeski, 2009). Its expression has been associated with both the death and proliferation of human epithelial uterine cells (ectocervical, endocervical and endometrial) as a result of direct activation by ATP (Wang et al., 2004; 2005; Zhou et al., 2006). The presence of functional P2X7 has also been identified on human basal and squamous cell carcinomas (Greig at al., 2003). In contrast, functional P2X7 on epithelial cells of the gastrointestinal system has not been clearly characterized. Coutinho-Silva et al (2005) demonstrated that ATP and BzATP induced death of the human colon epithelial carcinoma cell lines, HCT-8 and Caco-2 in a manner characteristic of P2X7 activation but a direct role for P2X7 in this process was not established. Data presented in this chapter demonstrates P2X7 agonists fail to induce ethidium⁺ uptake and death in HCT-8 and Caco-2 cells suggesting that these cells fail to express functional P2X7.

The lack of functional P2X7 in these cell lines could not be attributed to a technical deficiency in the ethidium⁺ uptake assay, as both the P2X7 agonists, ATP and BzATP induced high levels of ethidium⁺ uptake into RPMI 8226 cells. Moreover incubation of HCT-8 cells with increasing concentrations of IFN-γ (Welter-Stahl et al., 2009). failed to increase the ability of BzATP to induce ethidium⁺ uptake or increase cell-surface P2X7 expression.

However, since ATP-induced ethidium⁺ uptake assays measure P2X7 pore formation but not P2X7 channel opening (Wiley et al., 1998), we cannot exclude the possibility that the epithelial carcinoma cell lines tested in my study express low levels of the P2X7 channel, but lack the capacity to open the P2X7 pore. Consistent with this, Coutinho-Silva et al (2005) demonstrated that both ATP and BzATP can induce Ca²⁺
fluxes into HCT-8 and Caco-2 cells, and that the P2X7 antagonists, KN-62 and oxidised ATP, could partially impair these responses. Our laboratory however lacked the techniques to explore Ca\(^{2+}\) fluxes in HCT-8 and Caco-2 cells, but was considered not worth pursuing due to a lack of functional P2X7 responses with the MTT assay. Moreover, neither ATP nor BzATP could induce ethidium\(^{+}\) uptake in HCT-8 or Caco-2 cells in KCl or sucrose medium nominally free of both Na\(^{+}\) and Ca\(^{2+}\) ions, both of which can partially impair P2X7 function (Wiley \textit{et al.}, 1993; Michel \textit{et al.}, 1999; Stevenson \textit{et al.}, 2009).

Use of the MTT assay was unable to confirm the presence of functional P2X7 on HCT-8 and Caco-2 cells, and was unable to replicate the findings of Coutinho-Silva \textit{et al.}, 2005). These authors showed that low concentrations of ATP (10 µM) stimulated proliferation and high ATP concentrations (1 mM) induced death in HCT-8 and Caco-2 cells. In contrast, data in this chapter showed that ATP (1 mM) and the most potent P2X7 agonist, BzATP (200 µM) failed to induce significant levels of death in either of the cell lines. Reasons for these differences between the studies in this chapter to that of Coutinho-Silva (2005) are not readily apparent. Cells were maintained in identical culture medium, except that Caco-2 cells were maintained in medium containing 20% foetal calf serum according to the cell supplier's instruction. Moreover, the MTT assay employed was the same in both studies. Although performing the MTT assay with a more conventional step, i.e. not substituting the culture medium before MTT addition, I also failed to observe ATP- or BzATP-induced cell death. Moreover, this same MTT assay could be used to demonstrate that ATP and BzATP could induce death of the RPMI 8226 cell line, and that this ATP-induced cell death could be blocked by the P2X7 antagonists, KN-62 and A-438079 (Appendix I). One possible explanation for the
differences between my study and that of Coutinho-Siva and colleagues is the origin of
the epithelial cell lines. Coutinho-Silva et al (2005) obtained HCT-8 and Caco-2 cells
from the American Tissue Culture Collection, while the cells obtained in my study were
from the European Collection of Cell Cultures. Another possible explanation between
these two studies is that the HCT-8 and Caco-2 cell lines used in my study, expressed
higher amount of a P2X7 variant, which may inhibit P2X7 trafficking or function,
compared to the cell lines used by Coutinho-Silva et al (2005). However, Coutinho-
Silva and colleagues (2005) did not report immunoblotting data in their study, making
any direct comparisons difficult. Nevertheless, the relatively high amounts of a non-
glycosylated P2X7 (68 kDa) and a possible 42 kDa P2X7 variant, compared to the full-
length, glycosylated P2X7 (75 kDa) support this possible explanation. As discussed
below however this 42 kDa is not the P2X7-j variant and it will be of future importance
to characterise this band further. Coutinho-Silva et al (2005) found that the time course
of ATP-induced cell death was much longer (24-48 h) in HCT-8 cells compared with
other cells such as macrophages and dendritic cells (6 h) that express P2X7 at higher
levels (Coutinho-Silva et al., 1999; 2001). Therefore, longer ATP incubation times or
even higher concentrations of ATP could be considered with the HCT-8 and Caco-2
cells in our laboratory to further exclude the determine if these cell lines express
functional P2X7 and are sensitive to P2X7-induced cell death.

Immunolabelling with an anti-P2X7 mAb revealed the presence of low levels of
cell-surface P2X7 on HCT-8 and Caco-2 cells. Immunoblotting demonstrated that full-
length P2X7 was present in low amounts. Full length P2X7 (75 kDa) was present only
as a faint band in both HCT-8 and Caco-2 cells, which corresponds to the weak
expression of P2X7 mRNA in HCT-8 and Caco-2 cells (Coutinho-Silva et al., 2005).
Cancer epithelial cells express high levels of micro-RNAs miR-150 and miR-186 which stimulates degradation of the P2X7 transcript (Zhou et al., 2008), providing a possible reason for low P2X7 protein in HCT-8 and Caco-2. Alternatively, over-expression of the epidermal growth factor receptor may reduce P2X7 expression in HCT-8 and Caco-2 cells (Wang et al., 2005; Sibilia et al., 2007). Strong bands reactive with anti-P2X7 Ab were detected at 68 and 42 kDa. The 68 kDa band was most likely the non-glycosylated form of P2X7, while the 42 kDa band was most probably an unknown P2X7 variant or a breakdown product. This 42 kDa band was not however the P2X7-j variant, also 42 kDa in size and found in cancer epithelial cells (Feng et al., 2006), as this variant lacks the C-terminus and hence the peptide sequence (residues 576-595) to which the anti-P2X7 Ab was raised against. Our findings relating to HCT-8 and Caco-2 cells corresponds to findings in HeLa cells in which P2X7 mRNA and P2X7 protein was detected but also in which ATP did not elicit pore opening nor induce apoptosis, the two hallmarks of functional P2X7 expression (Welter-Stahl et al., 2009).

Data in this chapter (and Appendix I) confirms that the human multiple myeloma RPMI 8226 myeloma cell line express functional P2X7. Our laboratory has previously shown that these cells express cell-surface P2X7 by immunofluorescence labelling and flow cytometry (Farrell, 2008). The P2X7 immunoblotting data, as well as the immunofluorescence labelling data, presented in this chapter and the P2X7 RT-PCR data (chapter 3) confirms the presence of P2X7 in these cells. Moreover, previous data from our laboratory, has shown that P2X7 activation induces ethidium+ uptake, CD23 shedding and cell death in these cells, with the latter detected by Annexin-V binding and 7AAD uptake to detect phosphotidylserine exposure and loss of membrane integrity (Farrell, 2008). Again the ethidium+ uptake data (this chapter) and the MTT results
(Appendix A1.2) confirm that P2X7 activation can induce cation uptake and cell death in RPMI 8226 cells. Others have reported that P2X7 activation can induce cation uptake and cell death in normal and chronic lymphocytic leukemia (CLL) and B-lymphocytes (Adinolfi et al., 2002; Wiley et al., 2002; Gu et al., 2000). However the physiological and pathophysiological roles of P2X7 in normal and malignant B-lymphocytes remain obscure. To the best of our knowledge there have been no reports of defects in B-lymphocyte mediated responses in P2X7 knockout mice (Chessell et al., 2005; Solle et al., 2001). Moreover, defects in B-lymphocyte function have not been attributed to altered disease outcomes in models of inflammatory or immune-mediated disorders in these mice (Chen and Brosnan, 2006). Both enhanced P2X7-induced cell proliferation, due to high P2X7 amounts (Adinolfi et al., 2002), and defects in P2X7-induced cell death, due to the Glu\textsuperscript{496}Ala loss-of-function polymorphism (Wiley et al., 2002; Thunberg et al., 2002), have been postulated to alter disease outcomes in CLL (Virgilio and Wiley, 2002), however neither hypothesis has been substantiated by additional data (Sellick et al., 2004). Moreover, the Glu\textsuperscript{496}Ala polymorphism is not associated with disease outcomes in multiple myeloma (Paneesha et al., 2006). However, it should be noted that this loss-of-function polymorphism reduces P2X7 function by approximately 50% in heterozygous dosage (Gu et al., 2001), but does not inhibit P2X7 channel function (Boldt et al., 2003) nor completely impairs P2X7 function in homozygous dosage (Sluyter et al., 2004). Thus, the possibility remains that P2X7 may still play a role in B-lymphocyte malignancies independently of this polymorphic variation.

In conclusion, this study demonstrates that P2X7 agonists do not induce ethidium\textsuperscript{+} uptake into or death of the epithelial colon carcinoma cell lines, HCT-8 and
Caco-2. It will be of future interest to characterise the presence of the potential 42 kDa P2X7 variant f in these cell lines.
Chapter 5: P2X7 receptor activation induces death of myeloid leukemic KG-1 cells

5.1 Results

5.1.1 KG-1 cells express P2X7

KG-1 cells are a human myeloid leukemic cell line which can be differentiated into macrophages and dendritic cells (Baroni et al., 2007), however it is not known if this cell line expresses functional P2X7. Therefore, to determine the presence of P2X7 on the KG-1 cell line, cells were labelled with an anti-P2X7 monoclonal antibody (mAb) and examined by flow cytometry. As previously (Chapter 4), RPMI 8226 myeloma cells were used as a positive control. RPMI 8226 cells were found to express significantly high levels of P2X7 on the cell-surface compared to the isotype control (mean fluorescence intensity, MFI of 24.9 ± 3.5 and 5.4 ± 1.0 respectively, P < 0.01; n = 3, Fig. 5.1A). KG-1 cells were also found to express significant levels of cell-surface P2X7 compared to the isotype control (MFI of 4.8 ± 0.3 and 1.6 ± 0.1 respectively, P < 0.01; n = 3, Fig. 5.1B), although the amount of cell-surface P2X7 was 6-fold lower compared to RPMI 8226 cells. However it should be noted that this comparison in P2X7 expression between cell types was based on fold differences relative to the respective isotype mAb labelling for each cell type, thus the comparisons are only semi-quantitative.
Fig. 5.1 KG-1 cells express cell-surface P2X7. (A) RPMI 8226 (positive control) and (B) KG-1 cells were labelled with Alexa Fluor 647-conjugated anti-P2X7 (solid line) or isotype control (shaded) mAb and 7-amino-actinomycin-D (7AAD), and the relative cell surface expression (MFI) determined by flow cytometry. Representative results from three experiments are shown.

To determine whether this low amount of P2X7 on KG-1 cells was due to low cell-surface P2X7 expression or non-specific binding of the anti-P2X7 mAb, immunoblotting was performed on whole cell lysates of RPMI-8226 and KG-1 cells. Immunoblotting with an anti-P2X7 polyclonal antibody (Ab) revealed the presence of a major band at 75 kDa in RPMI 8226 cells (Fig. 5.2), the predicted size of P2X7. The 75 kDa band was also present in KG-1 cell (Fig. 5.2)s, however the band intensity in these cells was much lower compared to RPMI 8226 cells.
Fig. 5.2 P2X7 protein is present in KG-1 cells. Whole lysates of RPMI 8226 and KG-1 cells were separated by electrophoresis, transferred to nitrocellulose membrane and probed with anti-P2X7 antibody. Molecular weight markers, detected using StrepTactin-HRP, are shown in the far left lane. Results representative of three experiments are shown.

To confirm the presence of P2X7 mRNA in KG-1 cells, RNA was isolated from KG-1 cells, as well as from RPMI 8226 cells and analysed by RT-PCR. A PCR product of 544 bp was observed in both cell lines (Fig. 5.3) and this product corresponded to the predicted size of P2X7. Similar to the pattern observed with immunofluorescence labelling and immunoblotting, the expression of P2X7 mRNA in KG-1 cells was much lower compared to that of RPMI-8226 cells.
**Fig. 5.3** P2X7 mRNA is present in KG-1 cells. P2X7 mRNA from RPMI 8226 and KG-1 cells were amplified by RT-PCR, the products separated on a 2% agarose gel and visualised using ethidium bromide staining. Results representative of three experiments are shown.

### 5.1.2 KG-1 cells express functional P2X7

To determine if KG-1 cells express functional P2X7, cells were incubated in NaCl medium in the presence or absence of ATP or the most potent P2X7 agonist, 2(3′-O-(4-benzoylbenzoyl) adenosine 5′triphosphate (BzATP), for up to 30 min and the relative levels of ethidium\(^{+}\) uptake were measured at 5 min intervals by flow cytometry. ATP failed to induce significant levels of ethidium\(^{+}\) uptake in KG-1 cells at all time points compared to basal levels at corresponding time points (Fig. 5.4). In contrast, BzATP induced significant levels of ethidium\(^{+}\) uptake at 15, 20 and 30 min compared to corresponding basal levels (Fig. 5.4). BzATP did not induce significant ethidium\(^{+}\) uptake at earlier time points (Fig. 5.4). The highest level of BzATP-induced ethidium\(^{+}\) uptake was at 30 min, although this was found to be more variable than at 20 min. Thus, the 20 min time point was used for subsequent studies.
**Fig. 5.4** BzATP but not ATP induces ethidium\(^+\) uptake into KG-1 cells. KG-1 cells in NaCl medium containing 25 µM ethidium\(^+\) were incubated at 37ºC for over 30 min in the absence (basal) or presence of 1 mM ATP or 200 µM BzATP. Incubations were stopped by addition of MgCl\(_2\) medium and centrifugation and the ethidium\(^+\) uptake (MFI) was determined by flow cytometry. Results are shown as mean ± SD (n = 3); *P < 0.05 and **P < 0.01.

As mentioned previously (Section 4.1.3), the potency of BzATP for P2X7 is approximately 20-fold higher in sucrose medium compared to NaCl medium (Michel et al., 1999), and that the maximal response and efflux rates of the P2X7 receptor to BzATP and ATP are higher in KCl medium as compared to NaCl medium (Wiley et al., 2004; Stevenson et al., 2009). Also as discussed previously (Section 4.1.3), Ca\(^{2+}\) was omitted from the sucrose medium. Therefore, both ATP- and BzATP-induced ethidium\(^+\) uptake in to KG-1 cells was examined in cells resuspended in KCl medium or sucrose medium (without Ca\(^{2+}\)). NaCl medium was included as a comparison. As above (Fig. 5.4), ATP failed to induce significant ethidium\(^+\) uptake into cells in NaCl medium compared to basal levels (Fig. 5.5A). However, BzATP also failed to induce significant ethidium\(^+\) uptake (Fig. 5.5A) contrasting previous results (Fig. 5.4). In contrast, both
ATP and BzATP induced significant ethidium\(^+\) uptake in KCl and sucrose medium compared to basal levels of ethidium\(^+\) uptake in their respective media (Fig. 5.5B,C). Moreover, in either of these media BzATP-induced ethidium\(^+\) uptake was approximately two-fold higher than ATP-induced ethidium\(^+\) uptake.

**Fig. 5.5** ATP and BzATP induce ethidium\(^+\) uptake in KCl and sucrose medium but not in NaCl medium. KG-1 cells in NaCl or KCl medium or sucrose medium (without Ca\(^{2+}\)) containing 25 µM ethidium\(^+\) were incubated at 37ºC for 20 min in the absence (basal) or presence of 1 mM ATP or 200 µM BzATP. Incubations were stopped by addition of MgCl\(_2\) medium and centrifugation and the ethidium\(^+\) uptake (MFI) was determined by flow cytometry. Ethidium\(^+\) uptake is expressed as MFI. Results are shown as mean ± SD (n = 3); *P < 0.05, **P < 0.01 and ***P < 0.001.

### 5.1.3 ATP induces ethidium\(^+\) uptake in a concentration dependant manner in KG-1 cells

To determine if the ATP-induced ethidium\(^+\) uptake into KG-1 cells was mediated by P2X7, KG-1 cells in sucrose media (without Ca\(^{2+}\)) were first incubated with increasing concentrations of ATP and the relative amounts of ethidium\(^+\) uptake were measured. ATP induced ethidium\(^+\) uptake in KG-1 cells in a concentration-dependent manner, with a maximal uptake at 100 µM ATP and with an EC\(_{50}\) of 8.7 (±...
2.3) μM (Fig. 5.6). These values are approximately 3- and 10-fold lower respectively, than those obtained for recombinant P2X7 in transfected cells (Donnelly-Roberts, 2009) and native P2X7 in primary cells and cell lines (Jarvis and Khakh, 2009).

**Fig. 5.6** ATP induces ethidium\(^{+}\) uptake in a concentration-dependant manner in KG-1 cells. KG-1 cells in sucrose medium (without Ca\(^{2+}\)), containing 25 μM ethidium\(^{+}\) were incubated at 37°C for 20 min in the presence of increasing ATP concentrations as indicated. Incubations were stopped by addition of MgCl\(_2\) medium and centrifugation, and the ethidium\(^{+}\) uptake determined by flow cytometry. Ethidium\(^{+}\) uptake is expressed as percent maximum response to 100 μM ATP. Results are shown as mean ± SD (n = 3).

5.1.4 P2X7 agonists as well as the non-P2X7 agonist, αβmeATP, induces ethidium\(^{+}\) uptake in to KG-1 cells

P2X7 is activated by BzATP and ATP and to a lesser extent by adenosine 5′-O-[3-thiotriphosphate] (ATP-γ-S) but not by Úβ-methyl ATP (ÚβmeATP), adenosine diphosphate (ADP) or uridine triphosphate (UTP) (Bianchi et al. 1999; Donnelly-Roberts et al., 2009; Ralevic and Burnstock 1998; Surprenant et al., 1996). Therefore to further explore the pharmacology of the nucleotide-induced ethidium\(^{+}\) uptake into KG-1 cells, cells in sucrose medium (without Ca\(^{2+}\)) were incubated in the absence or presence
of a panel of nucleotides, (each used at 100 µM) and ethidium\(^{+}\) uptake measured. Both ATP and BzATP induced the highest amounts of ethidium\(^{+}\) uptake in to KG-1 cells with amounts significantly greater than basal ethidium\(^{+}\) uptake (Fig. 5.7) ATP\(-\beta\)-S and ß-methyl ATP also induced significant amounts of ethidium\(^{+}\) uptake into KG-1 cells, but to a lesser extent than that of ATP or BzATP. ADP and UTP failed to induce significant ethidium\(^{+}\) uptake in KG-1 cells with amounts similar to the basal ethidium\(^{+}\) uptake (Fig. 5.7). Thus, as for the ATP concentration response, the agonist profile for ethidium\(^{+}\) uptake into KG-1 cells is atypical of P2X7 compared to this receptor in other cell types.

**Fig. 5.7** KG-1 cells exhibit an agonist profile atypical of P2X7. KG-1 cells in sucrose medium (without Ca\(^{2+}\)) containing 25 µM ethidium\(^{+}\) were incubated at 37\(^{\circ}\)C for 20 min in the absence or presence of ATP, BzATP, ß-methylATP ATP\(\beta\)S, ADP and UTP (all 100 µM) at 37\(^{\circ}\)C. Incubations were stopped by addition of MgCl\(_2\) medium and centrifugation, and the ethidium\(^{+}\) uptake (MFI) determined by flow cytometry. Results are shown as mean ± SD (n = 3); *P < 0.05 and **P < 0.001.
5.1.5 P2X7 antagonists impair ethidium\(^+\) uptake assay in KG-1 cells

To further determine if nucleotide-induced ethidium\(^+\) uptake into KG-1 cells may be mediated by P2X7, cells in sucrose medium were incubated in the presence of P2X7 antagonists, KN-62, A-438079 and AZ10606120, or DMSO or H\(_2\)O diluent control (as indicated). Pre-incubation of cells with 1 \(\mu\)M KN-62 or 0.1 \(\mu\)M AZ10606120 significantly inhibited 0.1 mM ATP-induced ethidium\(^+\) uptake by 77\% and 100\% respectively compared to the corresponding controls (Fig. 5.8A). Similarly A-438079 significantly inhibited ATP-induced ethidium\(^+\) uptake in KG-1 cells by 100\% to the corresponding control (Fig. 5.8B). Basal ethidium\(^+\) uptake either in the presence of each antagonist or diluents control was similar (Fig. 5.8). These results indicate that ATP-induced ethidium\(^+\) uptake involved P2X7 activation.

**Fig. 5.8** P2X7 antagonists impair ATP induced ethidium\(^+\) uptake in KG-1 cells. KG-1 cells in sucrose medium (without Ca\(^{2+}\)) were pre-incubated at 37\(^\circ\)C for 15 min in (A) the presence of DMSO diluents, 1 \(\mu\)M KN-62, or 0.1 \(\mu\)M AZ10606120; (B) the presence of H\(_2\)O diluent or 10 \(\mu\)M A-438079, and (A, B) then with 25 \(\mu\)M ethidium\(^+\) in the absence (basal) or presence of 100 \(\mu\)M ATP at 37\(^\circ\)C for 20 min. Incubations were stopped by addition of MgCl\(_2\) medium and centrifugation, and ethidium\(^+\) uptake (MFI) determined by flow cytometry. Results are shown as mean ± SD (\(n = 3\)); *\(P < 0.05\) and ***\(P < 0.001\).
5.1.6 ATP reduces numbers of KG-1 cells

Next the MTT assay was used to determine if ATP induces death of KG-1 cells. The cytotoxic compound, 5,7-dibromo-N-(p-hydroxymethylbenzyl)isatin (isatin) (Vine et al., 2007) was used as a positive control. KG-1 cells in the absence or presence of ATP or isatin for 24 or 48 hours, and the relative cell number (expressed as absorbance) was determined. Incubation of KG-1 cells in the presence of 100 µM ATP or 3 µM isatin for 24 h significantly reduced the cell numbers by 44% and 66% respectively compared to cells incubated in the absence of agonist (Fig. 5.9). ATP- and isatin-induced cell death was more pronounced at 48 h, wherein a 51% and 61% reduction in cell numbers was observed compared to the untreated KG-1 cells (Fig. 5.9).

**Fig. 5.9** ATP reduces numbers of KG-1 cells. KG-1 cells in complete culture medium were incubated in the absence (basal) or presence of 100 µM ATP or 3 µM isatin at 37°C/5%/CO₂ for 24 hr or 48 hr. MTT was added during the final 4 hr, before overnight incubation with solubilisation solution at 37°C. Absorbances were then read at 550 nm and 690 nm. Results are mean absorbances (A₅₅₀nm - A₆₉₀nm) ± SD of (n = 3); *** P < 0.001.
5.1.7 P2X7 antagonists impair the ATP-induced reduction of KG-1 cell numbers

Next to determine whether the reduction in cell numbers by ATP was mediated by P2X7, KG-1 cells were pre-incubated in either the presence of the P2X7 antagonists, KN-62, AZ10606120 or A-438079, or DMSO or water diluent control (as indicated) for 24 or 48 h, and the relative cell number measured using the MTT assay. Similar to above (Fig. 5.9), 24 or 48 h incubation with ATP reduced KG-1 cell numbers (Fig. 5.10A,B). Pre-incubation of KG-1 cells with 1 μM KN-62 significantly inhibited the ATP-induced reduction in relative cell numbers by 68% and 78% at 24 and 48 h respectively (Fig. 5.10A). Similarly pre-incubation with 0.1 μM AZ10606120 significantly inhibited the ATP-induced reduction in cell numbers by 64% and 80% at 24 and 48 h respectively (Fig. 5.10A). In the absence of ATP the cell numbers were similar to those in the presence of DMSO, KN-62 or AZ10606120 (Fig. 5.10A). In contrast, A-438079, in the absence of ATP significantly reduced cell numbers at 24 and 48 h respectively (Fig. 5.10A). This reduction in cell number by A-438079 was also significantly reduced in the presence of ATP (Fig. 5.10A). Thus, A-438079 could not be used to determine if P2X7 mediated the ATP induced reduction in cell numbers.
Fig. 5.10 P2X7 antagonists, KN-62 and AZ10606120 impair ATP-induced reduction in KG-1 cell numbers. KG-1 cells in complete culture medium were pre-incubated at 37°C for 15 min in (A) the presence of DMSO, 1 µM KN-62 or 0.1 µM AZ10606120 or (B) presence of H2O or 10 µM A-438079, and (A, B) then in the absence or presence of 100 µM ATP at 37°C/5%/CO2 for 24 or 48 hr. (A, B) MTT was added during the final 4 hr, before overnight incubation with solubilisation solution at 37°C. Absorbances were then read at 550 nm and 690 nm Results are mean absorbances (A550nm - A690nm) ± SD of triplicate wells; *P < 0.05, **P < 0.01 and ***P < 0.001. Results representative of three experiments are shown.
5.1.8 ATP induces death of KG-1 cells

As outlined in Section 4.1.8, the MTT assay is an indirect measure of cell death. Therefore, to determine if the reduction in KG-1 cell numbers observed above was a result of cell death or impaired cell proliferation, KG-1 cells were incubated in the absence or presence of ATP or isatin for 24 or 48 h, and the percent cell viability assessed by trypan blue exclusion. In addition, the cells were viewed under a light microscope for morphological analysis. Apoptotic cells were identified using morphological criteria, including condensation of nuclei and shrinkage of cytoplasm (Kroemer G., et al, 2005; 2009). Incubation of KG-1 cells with 0.1 mM ATP or 3 μM isatin for 24 h significantly increased the percentage of dead cells by 4- and 14-fold respectively (Fig. 5.11A). Similarly, 48 h incubation with ATP and isatin increased the percentage of dead cells by 5- and 14-fold respectively (Fig. 5.11A). Morphological analysis demonstrated the presence of apoptotic cells following all treatments (Fig. 5.11B). Apoptotic cells were identified on the basis of rounding up of cells, membrane blebbing, shrinkage, DNA fragmentation and condensation of the nuclei of cells. Untreated KG-1 cells however predominantly displayed typical cell morphology of a heterogeneously shaped cell population (Fig. 5.11B). In contrast, more apoptotic cells were visible in cultures incubated in the presence of ATP or isatin for 24 or 48 h (Fig. 5.11B)
Fig 5.11 ATP induces death of KG-1 cells. (A,B) KG-1 cells in complete culture medium were incubated in the presence and absence of 100 µM ATP or 3 µM isatin 37°C/5%/CO₂ for 24 or 48 hr. (A) Aliquots of cells were resuspended in an equal volume of 0.4% trypan blue solution for 3 min and counted in a haemocytometer. The total numbers of viable and dead cells were then estimated, and expressed as percentage of dead cells. Results are shown as mean ± SD (n = 3); ***P < 0.001. (B) KG-1 cells in the original plated wells were examined using a phase contrast microscope. Scale bar is equal to 10 µm. Results are representative of three experiments.
5.2 Discussion

Zhang et al., 2004 have previously shown that the KG-1 variant cell line, KG1a, express functional P2X7 but the presence of P2X7 on the parental cell line KG-1 had not been investigated. Results in this chapter demonstrate that the myeloid leukemic cell line, KG-1, express functional P2X7 with an atypical pharmacological profile. Multiple evidences established that these cells express functional P2X7. First, immunofluoresence labelling, immunoblotting and RT-PCR demonstrated that KG-1 cells express P2X7 protein and mRNA. Second, the P2X7 agonists, ATP and BzATP (Bianchi et al., 1999; Donnelly-Roberts et al., 2009), induced ethidium\(^+\) uptake into these cells. Third, the far-less potent P2X7 agonist, ATP-\(\gamma\)S (Suprenant et al., 1996; Wiley et al., 1993) also induced partial ethidium\(^+\) uptake, while neither ADP nor UTP, well known non-P2X7 agonists (Jarvis and Khakh, 2009), failed to induced ethidium\(^+\) uptake. Fourth, three different P2X7 antagonists, KN-62 (Gargett and Wiley, 1997), AZ10606120 (Michel et al., 2008) and A-438079 (Mc Garaughty et al., 2007) impaired the ATP-induced ethidium\(^+\) uptake into KG-1 cells. Finally, the ATP- and BzATP-induced ethidium\(^+\) uptake was impaired in the presence of extracellular Na\(^+\) ions, a well-known feature of P2X7 activation (Stevenson et al., 2009; Wiley et al., 1999). In contrast, KG-1 cells displayed other pharmacological characteristics atypical of P2X7 activation. First, ATP induced ethidium\(^+\) uptake at a maximal concentration of 100 \(\mu\)M and with an \(EC_{50}\) of 8.7 \(\mu\)M, which is at least 3- and 10-fold lower respectively than that observed for P2X7-mediated cation fluxes in transfected and native cells (Donnelly-Roberts et al., 2009; Jarvis and Khakh, 2009). Second, \(\beta\)-methyl ATP, another well known non-P2X7 agonist (Jarvis and Khakh, 2009), induced partial ethidium\(^+\) uptake.
The reason for the high potency of ATP and the partial effect of \( \text{\textit{\( \beta \)}} \)-methyl ATP resulting in the atypical pharmacological profile of P2X7 in KG-1 cells was not established. One obvious consideration was that the ATP stock used was not prepared properly. However a series of observations exclude this possibility. First, absorbance measurements of the 100 mM ATP stocks confirmed that these were prepared at the correct concentration (Pupovac, personal communication). Second, the 100 \( \mu \text{M} \) ATP-induced ethidium\(^+\) uptake (and cell death) was observed with two separate ATP stocks. Third, these same ATP stocks induced ethidium\(^+\) uptake into RPMI 8226 cells with an \( \text{EC}_{50} \) of 122 \( \mu \text{M} \) (Appendix A1.1; Pupovac and Foster, personal communication), which is typical of recombinant P2X7 (Donnelly-Roberts \textit{et al.}, 2009; Jarvis and Khakh, 2009). Another consideration was that the only used \( \text{\textit{\( \beta \)}} \)-methyl ATP stock contained contaminating ATP. Commercial nucleotide preparations can contain other contaminating nucleotides (Mahaut-Smith \textit{et al.}, 2000). While comparison of the corresponding data (Fig. 5.6 and 5.7) indicate that 1 \( \mu \text{M} \) ATP and 100 \( \mu \text{M} \) \( \text{\textit{\( \beta \)}} \)-methyl ATP induced comparable amounts of ethidium\(^+\) uptake suggesting that as little as 1% contaminating ATP of the \( \text{\textit{\( \beta \)}} \)-methyl ATP stock may have contributed to the ethidium\(^+\) uptake observed with this synthetic analogue. In addition to using a second \( \text{\textit{\( \beta \)}} \)-methyl ATP stock, future studies should validate the purity of the \( \text{\textit{\( \beta \)}} \)-methyl ATP (example by high performance liquid chromatography) and the use ultra-pure \( \text{\textit{\( \beta \)}} \)-methyl ATP stocks either obtained commercially (if available) or made in-house. Nevertheless the \( \text{\textit{\( \beta \)}} \)-methyl ATP results do not account for the atypical results observed with ATP, which indicates that KG-1 cells express functional P2X7 with an atypical pharmacological profile.
A number of reasons for the atypical pharmacological profile of P2X7 in KG-1 cells can be proposed. First, P2X7 may be able to form heteromers with P2X4 (Guo et al., 2007) that may give rise to a receptor with P2X7-like characteristics. However the frequency or formation of these heteromers has been recently questioned (Nicke, 2009; Boumechache et al, 2009) and pharmacology data of these heteromers is still lacking. Thus, this appears to be an unlikely explanation for the atypical P2X7 of KG-1 cells; however future immunoblotting and RT-PCR studies should explore if other P2X subtypes are present in KG-1 cells. A second explanation may be that KG-1 cells express an alternate isoform of P2X7. A splice variant of P2X7, termed P2X7 (k), which contains an alternate exon 1 has been observed in rats and mice, and this isoform has increased sensitivity to ATP and BzATP (Nicke et al, 2009). Moreover, the P2X7 (k) isoform is 77 kDa which approximates to the size of the P2X7 reactive band observed in KG-1 cells. While the detection of the P2X7 transcript, spanning regions of exon 10 and 11, does not exclude the possibility that this transcript was the P2X7 (k) variant rather than the more commonly observed P2X7 isoform. Future studies, should include the potential detection of the P2X7 (k) isoform by RT-PCR. A final explanation may be that KG-1 cells express a variant of P2X7 arising from one or more single nucleotide polymorphisms (SNPs). A number of SNPs have been observed in the human P2RX7 gene (Fuller et al., 2009), however to the best of my knowledge none of these alter the pharmacology of the receptor except the Arg^{307}Gln SNP which totally ablates ATP or BzATP binding (Gu et al, 2004). In contrast, a number of mutations have been described which partially alter the pharmacological profile of murine P2X7 (Young et al, 2006). Future studies, should involve the complete sequencing of the P2RX7 gene in KG-1 cells.
ATP also induces the death of KG-1 cells by a receptor characteristic of P2X7. As for ethidium\(^+\) uptake significant cell death was observed at 100 \(\mu\)M, and this effect was greater than that observed at 1 mM ATP. Moreover, this ATP-induced cell death was impaired by KN-62 and AZ10606120. Morphological analysis indicated that the ATP-induced cell death was a result of apoptosis, with some cells being rounded up and shrunken, and evidence of membrane blebbing, and fragmentation. Future, studies should include the use of other apoptotic markers such as DNA fragmentation, phosphatidylserine exposure, loss of membrane integrity and caspase activation to conclusively establish that the ATP-induced KG-1 cell death is a result of apoptosis. Previous studies have shown that the KG-1 variant cell line KG1a also expresses functional P2X7, indicated by the presence of P2X7 protein and mRNA, and ATP- and BzATP-induced Ca\(^{2+}\) fluxes (Zhang et al., 2004). Further studies by this group have also shown that ATP and BzATP effectively inhibit cell growth which was completely blocked by the P2X7 antagonists, KN-62 and oxidised ATP. Moreover, Zhang et al (2009) demonstrated that ATP or BzATP induced ethidium\(^+\) uptake in sucrose but not NaCl medium, similar in formulation to the corresponding media used in my study. In contrast to my study however, 10- and 3-fold higher concentrations of ATP and BzATP respectively were required to induce either YO-PRO-1\(^{2+}\) uptake or cell death in KG1a cells, compared to similar processes in KG-1 cells. This ability of low concentrations of ATP to induce cell death in KG-1 cells however may be of physiological significance, as cells are more likely to be exposed low (100 \(\mu\)M) rather than high (1 mM) ATP concentrations \textit{in vivo}. Therefore further studies are required to characterise the pharmacology of P2X7 on KG1a cells as shown in this chapter.
In contrast to KN-62 and AZ10606120, the P2X7 antagonist A-438079 (in the absence of ATP) reduced KG-1 cell numbers as determined by the MTT assay. Of note, A-438079 did not affect basal ethidium+ uptake into KG-1 cells (Fig. 5.8). The reason for this effect of A438079 in the MTT assay appears to be unique to KG-1 cells, as previous data in our laboratory has failed to observe a similar effect with this compound in RPMI 8226 cells (Appendix A1.3) or with murine erythroleukemia cells (Constantinescu et al, 2010). Thus, prolonged incubation (24-48 h) with A-438079 either induces KG-1 cell death or prevents KG-1 cell proliferation. Future, studies could include the use of the trypan blue exclusion assay and morphological analysis to help delineate between these two mechanisms. Nevertheless the mechanism seems to be independent of P2X7 as neither KN-62 nor AZ10606120 reduced KG-1 cell numbers. It should be noted that ATP partially prevented the effect of A-438079 on KG-1 cells. Although these results are complicated by the cytotoxic role of ATP on KG-1 cells (established above), they imply that ATP may be acting as a competitive inhibitor of A-438079, and thus preventing A-438079 binding to its target molecule. The nature of this target molecule remains undefined, but may include a P2 receptor present on KG-1 but not on RPMI 8226 or MEL cells, although the species difference may also explain the non-cytotoxic effect of A-438079 on this latter cell type.

Isatin compounds and their derivatives are known to inhibit cell proliferation and stimulate apoptosis (Teeyapant et al., 1993; Nagle et al., 2004). Vine et al (2007) studied the cytotoxic effect of a range of substituted isatin compounds on leukemic and lymphoid cell lines. They found that these compounds were more sensitive to leukemic and lymphoma cell lines as opposed to breast, prostate, and colorectal carcinoma cell lines. The mode of action of the most potent compounds among them was characterised.
The compound, 5,6,7-tribromoisatin, was found to be anti-proliferative and activated the effector caspases, caspase-3 and caspase-7 in a dose-dependent manner. Romagnoli et al (2009) more recently have characterized a novel series of alpha-bromoacyryloyl N-substituted isatin analogues that were also found to be anti-proliferative towards the human myeloid leukemia HL-60 and U-937 cells, as well as human lymphoid leukemia MOLT-3 cells. These analogues caused a rapid release of cytochrome c from mitochondria into the cytosol and subsequent caspase activation involving caspase-3, to cleave poly(ADP-ribose) polymerase (PARP) followed by cell death. Although the mechanisms of isatin-induced death of KG-1 cells was not fully elucidated, results in this chapter support a role for isatin compounds in inducing death of myeloid leukemic cells. Collectively, these findings suggest isatins are useful compounds to study apoptotic events in cancer cells and are a potential target for anti-cancer drug development.

In conclusion, KG-1 myeloid leukemic cells express functional P2X7 which display an atypical pharmacological profile. Moreover activation of this receptor mediates death of KG-1 cells. Thus KG-1 cells maybe a useful cell line model to study atypical P2X7 and ATP-induced cell death.
Chapter 6: General Discussion

The general aim of this project was to study the expression and function of the P2X7 receptor in human myeloid leukemic and epithelial malignant cancer cell lines. My study (Chapter 3) confirmed that the myeloid leukemic THP-1 cell line does not express functional P2X7 except when differentiated with interferon-gamma (IFN-γ) and lipopolysaccharide (LPS), as originally shown (Humphreys and Dubyak, 1998). My study also confirmed that transforming growth factor-beta1 (TGF-β1) prevented the up-regulation of P2X7 expression and function by IFN-γ and LPS in THP-1 monocytes as previously shown (Tran, 2007; Georgiou and Sluyter, personal communication). Subsequent data within this thesis showed that TGF-β1 prevented the up-regulation of P2X7 by preventing P2X7 transcription and subsequent translation. Moreover, this effect of TGF-β1 was not due to a general impairment of THP-1 cell differentiation by IFN-γ and LPS as TGF-β1 did not prevent the up-regulation of the differentiation markers, CD14 and MHC class II. In contrast to previous work that suggested the presence of functional P2X7 on the epithelial colon carcinoma cell lines, HCT-8 and Caco-2 (Coutinho-Silva et al., 2005), my study (Chapter 4) demonstrated that both these cell lines expressed low levels of non-functional P2X7 receptors and indicated the presence of a possible P2X7 variant. My study also established the presence of functional P2X7 on the myeloid leukemic KG-1 cell line, but which displayed an atypical pharmacological profile being ~10-fold more sensitive to adenosine 5' triphosphate (ATP) and sensitive to the non-P2X7 agonist, Úβ-methyl adenosine 5' triphosphate (ÚβmeATP). Finally, my study (Chapters 3-5) also confirmed the presence of high levels of functional P2X7 in the multiple myeloma RPMI 8226 cell line cell line as previously observed (Farrell, 2008).
The myeloid leukemic THP-1 cell line has become a well-established model to study the expression and function of P2X7 following differentiation with IFN-γ and LPS, or with 12-myristate 13-acetate (PMA) (see Section 1.3). Undifferentiated THP-1 cells however express negligible amounts of functional P2X7 (Humphreys and Dubyak, 1998; Chapter 3). On the other hand, the myeloid leukemic KG-1 cell line express low amounts of functional P2X7, albeit with an atypical pharmacological profile, when studied in sucrose but not NaCl medium (Section 5.1.2). Of note undifferentiated THP-1 cells express low amounts of cell-surface P2X7 and P2X7 mRNA (Section 3.1.1), similar to that of KG-1 cells (Section 5.1.1). Thus, it will be of future interest to determine if ATP or the most potent P2X7 agonist, BzATP, can induce ethidium⁺ uptake into undifferentiated THP-1 cells in sucrose medium. Conversely, given the capacity of KG-1 cells to differentiate into macrophage-like cells (Auwerx et al., 1991; Mary and Koeffler, 2008) or dendritic cells (Baroni et al. 2007), this cell line may be useful in exploring the modulation of P2X7 during macrophage or dendritic cell differentiation or in response to various inflammatory stimuli, as previously conducted elsewhere and in my study with THP-1 cells.

The multiple myeloma RPMI 8226 cell line expresses high level of functional P2X7 (Farrell, 2008). This cell line was used as a positive control in my study of myeloid leukemic and epithelial colon carcinoma cell lines. Cell-surface P2X7 was ~16- and ~6-fold lower on the myeloid leukemic THP-1 and KG-1 cell lines compared to the RPMI 8226 cells (Sections 3.1.1 and 5.1.1). However, differentiation of THP-1 cells with IFN-γ and LPS over three days raised cell-surface P2X levels, similar to those observed in RPMI 8226 cells (Section 3.1.1 and 4.1.1). Of note however, P2X7-induced ethidium⁺ uptake was ~3-fold greater in RPMI 8226 cells than in differentiated THP-1
cells (Sections 3.1.2 and 4.1.2) despite similar amounts of cell-surface P2X7 expression. 

P2RX7 genotyping, via PCR and restriction enzyme digestion (Eslick et al., 2009), revealed that THP-1 cells but not RPMI 8226 cells are heterozygous for the Glu\textsuperscript{496}Ala loss-of-function polymorphism (Skarratt, Farrell, Sluyter, unpublished data). This polymorphic variation provides the most likely explanation for the difference in P2X7 function between these two cell lines despite similar cell-surface P2X7 expression.

Similar to THP-1 and KG-1 cell lines, the epithelial colon carcinoma HCT-8 and Caco-2 cell lines expressed ~9-fold less cell-surface P2X7 (Section 4.1.1) compared to RPMI 8226 cells (Section 3.1.1). Immunoblotting data (Sections 3.1.1, 4.1.1 and 5.1.1) demonstrated that each of the cell lines expressed full-length P2X7 (75 kDa), with amounts corresponding to amounts of cell-surface P2X7. In contrast, a potential P2X7 42 kDa variant was present in HCT-8 and Caco-2 cells (Section 4.1.1), but not in THP-1 (undifferentiated or differentiated) or KG-1 cells. This variant was postulated to be the main cause for the consistent lack of functional P2X7 responses in both epithelial cell lines (Section 4.2) despite extensive investigation including 3-day incubation of HCT-8 cells with IFN-\(\gamma\) (Section 4.1.4), which has been previously shown to up-regulate P2X7 in these cells (Welter-Stahl et al., 2009). However, as already noted for differentiated THP-1 and RPMI 8226 cells, the presence of single nucleotide polymorphisms, which can alter P2X7 trafficking or function (Fuller et al, 2009), provides another explanation for the variation in P2X7 expression and function between the cell lines. Alternatively, the variation between the cell lines studied may reflect differences in expression and function as observed between various primary leukocyte and epithelial cell types (Georgiou et al., 2005; Gu et al., 2000; Stevenson et al., 2009).
APPENDIX 1

Fig. A1.1 ATP induces ethidium$^+$ uptake in a concentration-dependant manner in RPMI 8226 cells. RPMI 8226 cells in NaCl medium, containing 25 μM ethidium$^+$ were incubated at 37°C for 5 min in the presence of increasing ATP concentrations as indicated. Incubations were stopped by addition of MgCl$_2$ medium and centrifugation, and the ethidium$^+$ uptake (MFI) determined by flow cytometry. Results are shown as mean ± SD ($n = 3$).
Fig. A1.2 ATP reduces numbers of RPMI 8226 cells. RPMI 8226 cells in complete culture medium were incubated in the absence (basal) or presence of 100 µM ATP or 200 µM BzATP at 37°C/5%/CO₂ for (A) 24 and (B) 48 hr. MTT was added during the final 4 hr, before overnight incubation with solubilisation solution at 37°C. Absorbances were then read at 550 nm and 690 nm. Results are mean absorbances (A₅₅₀nm - A₆₉₀nm) ± SD of (n = 3); ** P < 0.01, *** P < 0.001.

Fig. A1.3 P2X7 antagonists, KN-62 and A-438079 impair ATP-induced reduction in RPMI 8226 cell numbers. RPMI 8226 cells in complete culture medium were pre-incubated at 37°C for 15 min in (A) the presence of DMSO or 1 µM KN-62 (B) presence of H₂O or 10 µM A-438079, and (A, B) then in the absence or presence of 1 mM ATP at 37°C/5%/CO₂ for 24 hr. (A, B) MTT was added during the final 4 hr, before overnight incubation with solubilisation solution at 37°C. Absorbances were then read at 550 nm and 690 nm Results are mean absorbances (A₅₅₀nm - A₆₉₀nm) ± SD of triplicate wells; ***P < 0.001.
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