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Characterization of calcium-independent purinergic receptor-mediated apoptosis in hormone-refractory prostate cancer

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OBJECTIVE

To investigate the nature of purinergic signalling in hormone-refractory prostate cancer (HRPC) cells *in vitro*, as extracellular ATP inhibits the growth of HRPC *in vitro* via the activation of P2 purinergic receptors, and to characterize which P2 receptors subtypes and secondary mechanisms are involved.

MATERIALS AND METHODS

The effect of extracellular ATP on HRPC cell lines PC-3 and DU-145, and the normal prostate cell line PNT-2, were investigated. Reverse-transcription polymerase chain reaction was used to assess P2 purinergic receptors, which were pharmacologically characterized using various receptor agonists and antagonists. The effect of ATP on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was examined to asses its role in growth inhibition. The effect of combining ATP with the chemotherapeutic drug mitoxantrone was also assessed.

RESULTS

PC-3 cells expressed mRNA for $P2X_{4,5,7}$, $P2Y_{1,2,4,6}$; DU-145 cells expressed mRNA for $P2X_{4,5}$, $P2Y_{1,2,4,6,11}$; PNT-2 cells expressed mRNA for $P2X_{4,5,7}$ and $P2Y_{1,2,4,6,11}$. ATP (10^{-4} M) inhibited HRPC PC-3 cell growth by ≈90%, an effect partially inhibited by the nonselective P2 receptor antagonists pyridoxal-5′-phosphate-6-azophenyl-2′,4′ disulphonic acid (PPADS) and suramin. The order of potency of agonists was: adenosine 5′-O-(3 thiotriphosphate) > ATP > benzoyl benzoyl ATP >> 2-methylthio ATP. DU-145 cells responded similarly. Pharmacological profiling implicated $P2X_5$ and/or $P2Y_{11}$ receptors in the antineoplastic response in HRPC. ATP induced apoptosis in a $[Ca^{2+}]_i$-independent mechanism. ATP was significantly less effective on PNT-2 cells, which also had a different order of agonist potency. ATP combined with mitoxantrone in an additive manner in HRPC.

CONCLUSIONS

ATP effectively reduces growth of HRPC cells via calcium-independent apoptosis. Pharmacological profiling indicates $P2X_5$ and/or $P2Y_{11}$ receptors in this process, with a different functional purinergic receptor profile and sensitivity in normal vs cancer cells.

KEYWORDS

Apoptosis, ATP, cancer, prostate, purinergic signalling

INTRODUCTION

Hormone therapy is the primary treatment for metastatic prostate cancer. The response to androgen ablation is rapid, and although not curative, it is effective at reducing symptoms and sequelae of local tumour growth and metastatic tumour spread. About 20% of patients will fail to respond to first-line hormonal therapy [1]. In addition, patients who initially respond to hormone therapy eventually develop hormone-refractory prostate cancer (HRPC). Although the time taken for this to occur varies from patient to patient, once prostate cancer does becomes hormone refractory, it is difficult to treat and is usually fatal within 9–12 months [2].

Little is known about the control of growth in HRPC. In hormone-sensitive normal prostate and prostate cancer cells, androgen ablation leads to a sustained rise in intracellular calcium ion concentration ($[Ca^{2+}]_i$), and subsequent apoptotic cell death [3]. This response to androgen ablation is lost in hormone-refractory cells. However, studies have shown that modest elevations in $[Ca^{2+}]_i$ for sufficient time, can still induce apoptotic cell death in HRPC cells raising the possibility that alterations in calcium homeostasis could still be the key to apoptosis induction in HRPC [4].

ATP mediates a variety of biological functions including synaptic neurotransmission, smooth muscle contraction or relaxation, and exocrine or endocrine secretion. There is now increasing awareness that ATP, acting through specific receptors (P2 purinergic receptors) can have trophic actions on various cells including induction of cell proliferation, differentiation, migration and death [5].

Fang *et al.* [6] first reported that ATP could inhibit the growth of prostate cancer cell lines *in vitro* via the activation of P2 receptors. Studies by Jansenns and Boeynaems [7] further characterized this ATP-induced growth inhibition as being due to the activation of P2X receptor subtypes. However, the further differentiation of which specific

P2X receptor subtypes were involved, and their mechanism of action, remained unclear.

In the rapidly developing field of purinergic research, which is <40 years old, the discovery of more receptor subtypes and the development of more selective agonists and antagonists allows for more accurate assessment of the involvement of purinergic signalling in various biological processes. In this study, we undertook experiments to further investigate the nature of purinergic signalling in HRPC cells *in vitro*. For comparison we also investigated a normal prostate epithelial cell line, PNT-2. Using reverse- transcription (RT) PCR, we established which P2 receptor protein subtypes were present. Using various P2 receptor agonists and antagonists, we undertook the pharmacological characterization of these receptor subtypes, to assess which were functionally involved in growth inhibition. In particular, experiments were undertaken to ascertain the role of pro-apoptotic $P2X_7$ receptors in the observed cytotoxic response. Using apoptotic detection kits (annexin V and propodium iodide (PI)) we qualitatively and quantitatively assessed the contribution of apoptosis in the observed growth inhibition.

As ATP has been shown to increase $[Ca^{2+}]_i$ in various human cancer cell lines including colorectal cancer [8], oesophageal cancer [9] and breast cancer [10], we also investigated the effect of ATP on $[Ca^{2+}]_i$ in HRPC cells *in vitro* to determine the role of Ca^{2+} ions in any observed ATP-induced growth inhibition.

Given the poor traditional response of HRPC to cytotoxic chemotherapy, the final aspect of this study assessed the effect of combining ATP with the chemotherapeutic drug mitoxantrone *in vitro*.

MATERIALS AND METHODS

For the cell cultures, PC-3 and DU-145 HRPC cell lines, as well as the normal prostate cell line PNT-2 were obtained from the European Collection of Cell Cultures (Salisbury, Wilts, UK).

The PC-3 cells were grown in Ham's F12K medium with 2 mM L-glutamine supplemented with 7.5% fetal calf serum (FCS; Sigma Chemical Co., Poole, UK) and a 1% antibiotic solution (containing penicillin, streptomycin and amphotericin B; Sigma). The DU-145 cells were grown in minimum essential medium (MEM) containing 2 mM L-glutamine supplemented with 10% FCS, 1% nonessential amino acid solution (Sigma) and a 1% antibiotic solution. The PNT-2 prostate epithelial cells were grown in RMPI 1640 medium with 2 mM L-glutamine supplemented with 10% FCS and a 1% antibiotic solution. All cells were maintained in a humidified atmosphere at 37 °C containing 5% CO_2.

RT-PCR

Total RNA was extracted from the three cell lines in culture using the SV Total RNA Isolation system (Promega, WI, USA). RT and cDNA amplification for all the P2 receptors was carried out with a thermal cycler (Hybaid, UK) in a two-step protocol using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, UK). Every sample was further treated with amplification grade DNase I (Sigma) to remove any residual DNA present that could generate false-positive results. Briefly, 1 μg of total RNA was reverse transcribed using the $pd(T)_{12–18}$ as the first-strand primer at 42 °C for 30 min and the enzyme was denatured at 95 °C for 5 min The sequence specific primers (Life Technologies, NY, USA) for the P2X and P2Y receptors were then added to the reaction mixtures. The PCR cycling parameters were 95 °C for 30 s, 54–62 °C for 1 min (depending on the primer set being used), 72 °C for 1 min for 35 cycles, followed by a further stage of extension at 72 °C for 5 min The resulting PCR products were resolved in a 2% agarose gel containing ethidium bromide and observed under ultraviolet illumination. The product sizes for each of the primer sets used were as follows: $P2X_1$ 510 base pairs (bp); $P2X_2$, 355 bp; $P2X_3$ 695 bp; $P2X_4$ 295 bp; $P2X_5$ 595 bp; $P2X_6$, 520 bp; $P2X_7$ 674 bp; $P2Y_1$ 527 bp; $P2Y_2$ 637 bp; $P2Y_4$ 424 bp; $P2Y_6$ 364 bp; and $P2Y_{11}$ 410 bp. The RT-PCR experiments for each set of primers were repeated using at least three separate preparations of total RNA, from at least three separate cell cultures.

MEASURING THE EFFECT OF P2 RECEPTOR AGONISTS AND ANTAGONISTS ON CELL GROWTH

PNT-2, PC-3 or DU-145 cells were seeded at a density of 50 000 cells/mL in 24-well plates. After 24 h, the number of adherent viable cells in the control wells was recorded using the haemocytometer method of counting Trypan blue excluding cells.

ATP in increasing concentrations (10^{-7}–10^{-3} M: 12 per concentration) was added to each different row of wells. After 72 h, cell viability was assessed in both the control and treated wells. Experiments were repeated in the presence of the P1 receptor antagonist 8-thiophenyltheophylline (8-SPT, 10^{-6}–10^{-4} M; nine per concentration), the nonspecific P2 receptor antagonists pyridoxal-5′-phosphate-6-azophenyl-2′,4′ disulphonic acid (PPADS) and suramin (both 10^{-6}–10^{-4} M, nine per concentration) and the $P2X_7$ receptor antagonist 1-[N, O-bis (5-isoquinolinesulphonyl)-N-methyl-L-tryosyl]-4-phenylpiperazine (KN-62; 10^{-6}–10^{-4} M, nine per concentration).

Experiments were also repeated using the P2 receptor agonists adenosine 5′-O-(3 thiotriphosphate) (ATPγS), benzoyl benzoyl ATP (BzATP), 2-methylthio ATP (2-MeSATP), 2-methylthio ADP (2-MeSADP), uridine 5′-triphosphate (UTP), uridine diphosphate (UDP) and α,β-methylene ATP (α,β-meATP) (all 10^{-7}–10^{-4} M, nine per agonist concentration). All agonists and antagonists were obtained from Sigma.

ASSESSMENT OF $P2X_7$ LIGAND-GATED ION CHANNEL ACTIVATION

Cell permeabilization by activation of pro-apoptotic $P2X_7$ receptor related-membrane pores was assessed in all three cell lines by observing the differential uptake of the fluorescent dye lucifer yellow (LY) after treatment with ATP. In the absence of $P2X_7$-gated pore formation, cellular LY uptake is by internalization into large, discreetly visible intracellular vacuoles. By contrast, the presence of $P2X_7$-gated pores leads to the rapid uptake of LY visible throughout the entire cell cytoplasm and nuclear region. The cells were grown on coverslips and incubated in complete MEM medium containing LY at 5 mg/mL. The cells were incubated either with or without ATP 10^{-3} M for varying durations (15–60 min) at 15 min intervals (three for each) at 37 °C. After incubation, all cells were washed with PBS and immediately viewed using a Zeiss Axioplan fluorescence microscope with a dark field. Cells within the supernatant, and those still adherent to the coverslips were mounted on slides with a 'blinded' code.

ASSESSMENT OF ATP-MEDIATED APOPTOSIS

PC-3 cells grown on coverslips were incubated in media either with or without ATP 10^{-3} M (five for each). After 72 h, adherent cells and those within the supernatant were analysed separately for apoptosis induction using annexin V-fluorescein isothiocyanate and PI, stained according to the manufacturers protocol (Sigma). Supernatants and coverslips were mounted on slides with a 'blinded' code and viewed using a Zeiss fluorescence microscope with a dark field. The proportion of cells undergoing early apoptosis (i.e. staining positive for annexin V and negative for PI) were counted in three representative high powered fields from each sample.

INTRACELLULAR CALCIUM MEASUREMENTS

Fluorometric Imaging Plate Reader (FLIPR)

A FLIPR machine (Molecular Devices, UK) was used for all $[Ca^{2+}]_i$ measurements. Experiments were repeated in triplicate on each occasion, and on at least three separate occasions to assess for congruity of results.

Fluorescent-dye loading

HRPC cells were cultured in specialized 96-well multiplates and loaded with fluo 4-AM fluorescent dye. Experiments were performed in Hanks' balanced salt solution (HBSS) solution containing (mM); NaCl, 142; KCl, 5.6; $MgCl_2$, 1; $CaCl_2$, 2; Na_2HPO_4, 0.34; KH_2PO_4, 0.44; HEPES, 10; glucose, 5.6; buffered to pH 7.4 with NaOH. The Ca^{2+}-free HBSS had the same constituents as HBSS solution, but with no $CaCl_2$ and with EGTA 1 mM added to eliminate any possible calcium contamination.

PHARMACOLOGICAL CHARACTERIZATION

The cells were treated with various P2 receptor agonists at 10^{-4} M and 5×10^{-5} M (ATP, ATPγS, BzATP, 2-MeSATP, 2-MeSADP, UTP, UDP, α,β-meATP) either in the presence or absence of extracellular Ca^{2+}. Full dose–response curves were plotted for the most effective agonists, ATP and UTP (10^{-7}–10^{-3} M). In addition, the effect of adding UTP 10^{-4} M after ATP 10^{-4} M was assessed, to identify if different cellular mechanisms were involved in increasing $[Ca^{2+}]_i$ with these two agonists.

To assess the secondary mechanism involved in ATP induced changes in $[Ca^{2+}]_i$ experiments were repeated after incubation with the phospholipase C (PLC) inhibitor U73122 (5×10^{-5} M), or its inactive analogue U73343 (5×10^{-5} M) for 30 min.

ASSESSMENT OF THE EFFECT OF ATP ALONE AND IN COMBINATION WITH CHEMOTHERAPY

PC-3 cells were seeded at a density of 50 000 cells/mL in 24-well multiwell plates. The chemotherapeutic drug mitoxantrone (Sigma) was added to the experimental wells at increasing concentrations (10^{-9}–10^{-4} M; 12 per concentration) to obtain a dose–response curve. After 72 h of incubation, cell viability was assessed using the haemocytometer and Trypan blue exclusion. The experiments were then repeated with cells incubated either with mitoxantrone alone, or with a combination of mitoxantrone with a single addition of ATP at a concentration below its IC_{50} (10^{-4} M; 12 per combination).

Data are expressed as the mean (SEM). Cumulative response curves were plotted and compared using a two-way ANOVA or a two-tailed *t*-test, as appropriate.

To assess synergy between ATP and mitoxantrone the calculation for 'Bliss Synergy' according to the fractional product analysis was used. Cytotoxic fractions (CF) were calculated as 1 – surviving fraction of cells. The fractional product value was then calculated as: CF [combined therapy]/{CF [ATP] + CF [Chemotherapy alone] – (CF [ATP] × CF [Chemotherapy alone])}. Values >1 indicated Bliss synergism, values ≈equal to 1 indicated additivity, and values <1 indicated Bliss antagonism.

RESULTS

EXPRESSION OF P2 RECEPTOR MRNA

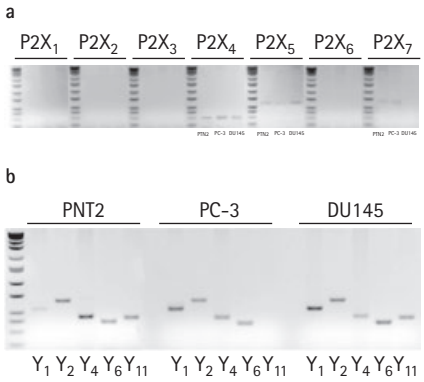
PC-3 cells expressed mRNA for $P2X_{4,5,7}$, $P2Y_{1,2,4,6}$; DU-145 cells expressed mRNA for $P2X_{4,5}$, $P2Y_{1,2,4,6,11}$; PNT-2 cells expressed mRNA for $P2X_{4,5,7}$ and $P2Y_{1,2,4,6,11}$.

None of the cells lines expressed mRNA for $P2X_{1,2,3\ or\ 6}$ receptors (Fig. 1).

THE EFFECTS OF P2 RECEPTOR AGONISTS AND ANTAGONISTS ON PROSTATE CANCER CELL GROWTH

At 24 h the number of viable PC-3 cells had increased to 60 000 cells/mL in the control

FIG. 1. Prostate cell line P2 receptor subtype mRNA as detected by RT-PCR (PNT-2, normal human prostate epithelium; PC-3, Gleason grade 4 prostate adenocarcinoma; DU-145, Gleason grade 2 prostate adenocarcinoma). (a) mRNA for P2X receptor subtypes. (b) mRNA for P2Y receptor subtypes.

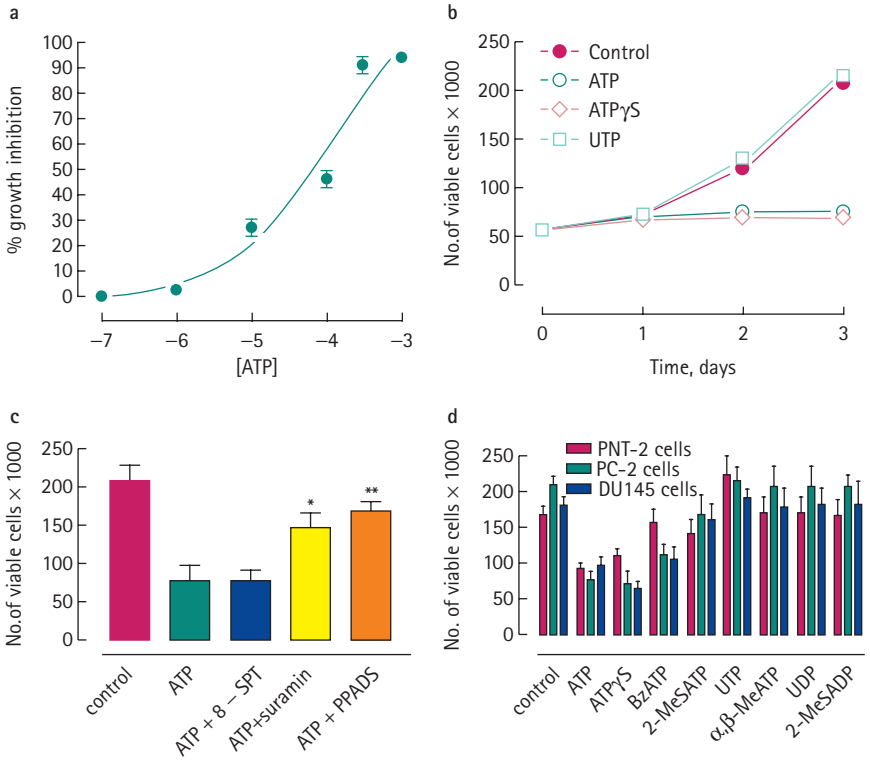


wells. After a further 72 h the cell numbers in the control wells increased to 206 000 cells/mL (i.e. the cell-doubling time was ≈36 h). The addition of exogenous ATP decreased cell growth in a dose dependent manner (IC_{50} 1.35×10^{-4} M ± 5.0×10^{-5} M) (Fig. 2a). The single addition of ATP 10^{-4} M inhibited cell growth by 45 (2.3)%. The daily addition of ATP 10^{-4} M inhibited cell growth by 88 (3.2)% (Fig. 2b). The addition of 8-SPT and KN-62 (10^{-6}–10^{-4} M) had no effect on ATP-mediated growth inhibition. The addition of the P2 receptor antagonists PPADS ($P = 0.06$) and suramin ($P < 0.05$) (all 10^{-4} M) partially inhibited the antineoplastic action of ATP (Fig. 2c).

The P2Y receptor agonists 2-MeSADP ($P2Y_1$), UTP ($P2Y_2$, $P2Y_4$) and UDP ($P2Y_6$) had no significant effect on growth inhibition of PC-3 cells, with lower concentrations (10^{-7}–10^{-6} M) of the former two actually increasing the number of viable cells (Fig. 2d). The P2X receptor agonist α,β-meATP ($P2X_1$, $P2X_3$) also had no significant effect on cell growth. The greatest reduction in cell viability was seen with the hydrolysis resistant P2 agonist ATPγS (Fig. 2b,d). The order of agonist potency was: ATPγS > ATP > BzATP >> 2-MeSATP.

The DU-145 cells responded similarly to the PC-3 cells in all aspects. One addition of ATP 10^{-4} M inhibited cell growth by 49 (2.7)% (IC_{50} 1.3×10^{-4} ± 3.7×10^{-5} M) (Fig. 3a). The daily addition of ATP 10^{-4} M inhibited cell growth by 91 (3.6)% (Fig. 3b). The DU-145 cells also

*FIG. 2. The effect of P2 receptor agonists and antagonists on growth of PC-3 cells. (a) Dose–response curve to ATP (IC_{50} 1.35 × 10^{-4} ± 5.0 × 10^{-5} M). (b) Effect of daily addition of various P2 receptor agonists (all 10^{-4} M) on cell growth over 72 h. (c) Effect of various P2 receptor antagonists (all 10^{-4} M) on ATP-mediated growth inhibition. (d) Comparison of the effect of various P2 receptor agonists (all 10^{-4} M) on PNT-2, PC-3 and DU-145 cells in vitro. All points are the mean (SEM) unless occluded by the symbol. *P < 0.05; **P < 0.001.*



exhibited the same response to the various P2 receptor agonists and antagonists, with the same order of agonist potency as previously observed (ATPγS > ATP > BzATP >> 2-MeSATP) confirming congruity of results between these two different HRPC cell lines (Figs 2d,3b).

However, the normal prostate epithelial cell line PNT-2 had a markedly different response to the different purinergic agonists. One addition of ATP 10^{-4} M only inhibited cell growth by 19 (3.2)% (IC_{50} 2.5 × 10^{-4} ± 2.5 10^{-5} M) (Fig. 3c).

The order of agonist potency was also different to that of the HRPC cells: ATP > ATPγS ≥ 2-MeSATP >> BzATP (see Figs 2d,3d), suggesting a different complement of functional receptors and a different sensitivity to P2 receptor-mediated growth inhibition. Also of note was the marked mitogenic effect of UTP on PNT-2 cells, compared with the little/no increase in cell growth in both HRPC cell lines.

ASSESSMENT OF CELL PERMEABILIZATION VIA $P2X_7$ LIGAND-GATED ION CHANNEL ACTIVATION

The $P2X_7$ receptor ligand-gated ion channel forms a cell membrane pore with a molecular threshold of ≈900 Da. Its presence allows otherwise impermeant markers, such as LY, to traverse the cell membrane easily after $P2X_7$ receptor activation. In the present experiments, the incubation of all three cell lines with ATP 10^{-3} M for durations of 15–60 min did not lead to an uptake pattern consistent with $P2X_7$ receptor activation, excluding apoptosis induction secondary to cell permeabilization by this pro-apoptotic receptor subtype (Fig. 4a).

ASSESSMENT OF ATP-MEDIATED APOPTOSIS

One addition of ATP 10^{-3} M reduced the adherent viable PC-3 cells by 66 (1.5)% after 72 h. No viable cells were present in the supernatant, with all cells staining positive with annexin V, or annexin V and PI, indicating an array of cells at different stages of apoptotic cell death. Staining of the remaining adherent cells showed significantly more annexin V-positive cells compared with the control, indicating an increase in early apoptosis (annexin V-positive cells; control 1.9 (0.16)% vs ATP-treated 4.8 (0.17)%: *t*-test $P < 0.001$; Fig. 4b)

INTRACELLULAR CALCIUM MEASUREMENTS

ATP 10^{-4} M induced a biphasic increase in $[Ca^{2+}]_i$ in both HRPC cell lines. Experiments repeated in the absence of extracellular Ca^{2+} induced a monophasic increase in $[Ca^{2+}]_i$, with a reduction of about half in the initial $[Ca^{2+}]_i$ rise (Fig. 5a). This ATP-induced response was dose-dependant (Fig. 5b). UTP 10^{-4} M induced a similar biphasic and monophasic increase in $[Ca^{2+}]_i$ in the presence and absence of extracellular Ca^{2+} (Fig. 5c). ATP and UTP were equipotent at inducing changes in $[Ca^{2+}]_i$, and addition of UTP after ATP (both 10^{-4} M) did not cause any further rise in $[Ca^{2+}]_i$ (Fig. 5d). The agonists 2-MeSATP, 2-MeSADP and α,β-meATP had no significant effect on $[Ca^{2+}]_i$ at concentrations of up to 5 × 10^{-4} M.

The PLC inhibitor U73122 (5 × 10^{-5} M) completely inhibited the ATP-induced increase in $[Ca^{2+}]_i$. An equipotent dose of the inactive analogue U73343 did not inhibit the ATP-induced change (Fig. 5e).

ASSESSMENT OF THE EFFECT OF ATP ALONE AND IN COMBINATION WITH CHEMOTHERAPY

One addition of ATP 10^{-4} M inhibited cell growth by 45%. Mitoxantrone reduced cell viability in a dose-dependent manner (IC_{50} 4.82 × 10^{-8} ± 5.91 × 10^{-9} M). Adding ATP to mitoxantrone significantly shifted the dose–response curve to the left (ANOVA, $P < 0.001$). Combined therapy reduced the dose at which half of the cells were killed by a factor of 10 (combined IC_{50} 6.61 × 10^{-9} ± 1.48 × 10^{-9} M). The additive effect of drug combination was statistically significant (IC_{50} mitoxantrone alone vs mitoxantrone + ATP: *t*-test, $P < 0.001$) (Fig. 6).

DISCUSSION

The cytotoxic effect of extracellular ATP has been investigated in different malignancies, although the exact mechanism of its action remains unclear. The pro-apoptotic $P2X_7$ receptor has been strongly implicated in this phenomenon, as has the effect of ATP on

FIG. 3. The effect of P2 receptor agonists and antagonists on the growth of DU-145 and PNT-2 cells. (a) Dose–response curve to ATP on DU-145 cells (IC_{50} $1.30 \times 10^{-4} \pm 3.7 \times 10^{-5}$ M). (b) Effect of daily addition of various P2 receptor agonists (all 10^{-4} M) on growth of DU-145 cells over 72 h. (c) Dose–response curve to ATP on PNT-2 cells (IC_{50} $2.5 \times 10^{-4} \pm 2.5 \times 10^{-5}$ M). (d) Effect of daily addition of various P2 receptor agonists (all 10^{-4} M) on the growth of PNT-2 cells over 72 h. All points are the mean (SEM) unless occluded by the symbol.

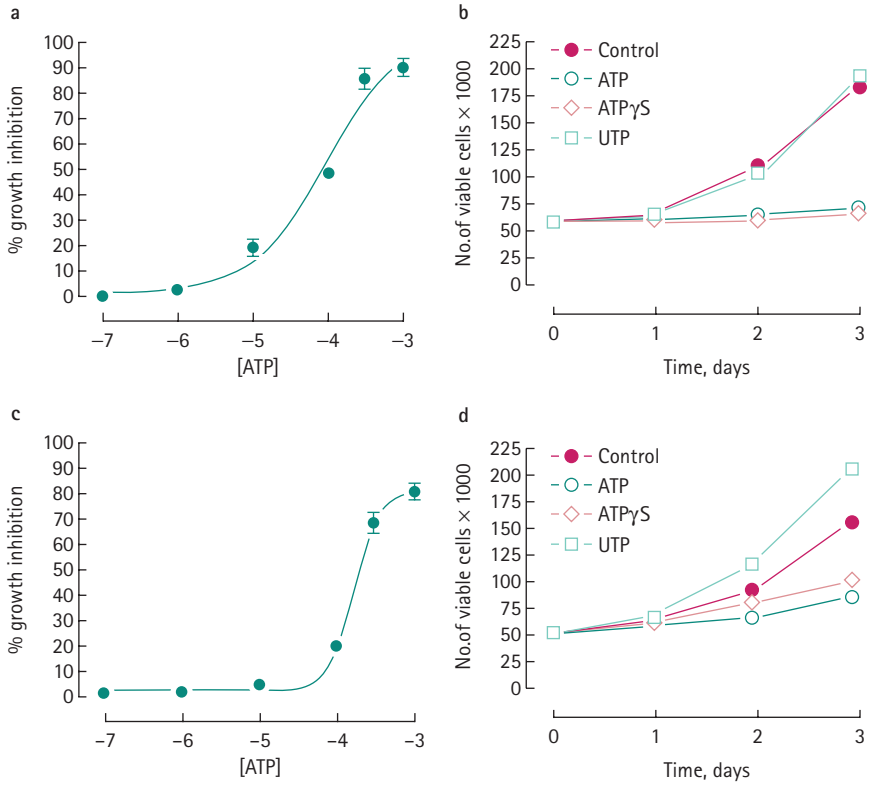
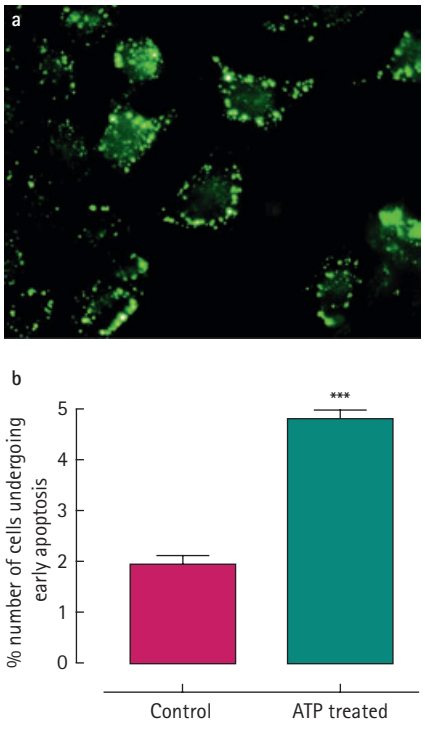


*FIG. 4. (a) Assessment of $P2X_7$ pore formation using LY. PC-3 cells were incubated with ATP 10^{-3} M at 37 °C for up to 1 h and assessed using fluorescence microscopy. LY entered the cells by pinocytosis only (discreet 'granular' appearance). No pore formation (typified by diffuse cellular uptake) was identified. (b) Bar chart showing the percentage of cells undergoing early apoptosis (positive staining for annexin V) in control and ATP-treated (10^{-3} M) PC-3 cells. All bars are the mean (SEM). ***P < 0.001.*



$[Ca^{2+}]_i$. Previous studies have already implied an antineoplastic action for ATP in HRPC cells acting via P2 receptors [6]. Further studies suggested that P2X receptor subtypes mediated this cytotoxic response, although the specific receptor subtypes and secondary mechanism involved remains uncertain [7]. The developing nature of purinergic research has allowed the characterization of more P2 receptor subtypes as well as the subsequent development of more selective agonists and antagonists for more specific assessment of purinergic signalling in biological systems. The aim of the present study was to investigate which specific P2 receptor subtypes were involved in the growth inhibition of HRPC cells given recent advances in purinergic research, and in particular to assess the role of the $P2X_7$ receptor and calcium ion homeostasis in the observed cytotoxic responses.

Using RT-PCR, we identified the presence of several different P2 receptor subtype mRNAs in the prostate. The normal prostate epithelial cell line PNT-2 expressed mRNA for $P2X_{4,5,7}$ and $P2Y_{1,2,6,11}$ receptors. HRPC PC-3 cells expressed the same profile, except that no $P2Y_{11}$ receptor mRNA was detectable. DU-145 cells also expressed the same profile, although no $P2X_7$ receptor mRNA was detected. The expression profiles for the cancer cell lines are in agreement with a previous report by Calvert *et al.* [11] that reported the expression of $P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$ and $P2Y_2$ receptor protein shown by immunohistochemistry in PC-3 cells.

Using pharmacological characterization, we assessed which of these receptors were functionally active in the inhibition of cell growth. In both HRPC cell lines, the P1 receptor antagonist 8-SPT had no effect on the ATP-induced response, showing that ATP was not acting via its breakdown product, adenosine. However, the nonselective P2 receptor antagonists, PPADS and suramin, reversed ATP-mediated growth inhibition, confirming both a key role for P2 receptors and excluding the involvement of the $P2X_4$ receptor in the control of cell growth, as these antagonists are ineffective at this receptor [12]. The $P2X_1$ and $P2X_3$ receptor agonist, α,β-meATP, had no significant effect on cancer cell growth, suggesting only minimal, if any, functional role for these receptor subtypes. The P2Y receptor agonists UTP ($P2Y_2$, $P2Y_4$) and UDP ($P2Y_6$) exerted no antineoplastic effect, excluding any role for these receptor subtypes in the observed growth inhibition. UTP had a mitogenic effect on both HRPC cell lines, contrary to the findings of other groups investigating human malignancies such as oesophageal and colon cancer [8,9], but in keeping with the recognized growth-stimulatory effects of the P2Y receptor subtypes as previously described [13]. The final order of agonist potency suggested a key central role for the $P2X_5$ receptor in the growth inhibition of HRPC cells, although

FIG. 5. (a) Biphasic and monophasic increases in $[Ca^{2+}]_i$ in PC-3 cells after adding ATP 10^{-4} M. (b) Dose–response curve of change in fluorescence ($[Ca^{2+}]_i$) in PC-3 cells after the addition of increasing concentrations of ATP. (c) Biphasic and monophasic increases in $[Ca^{2+}]_i$ in PC-3 cells after addition of UTP 10^{-4} M. (d) Biphasic increases in $[Ca^{2+}]_i$ in PC-3 cells after adding ATP 10^{-4} M. A second addition of UTP 10^{-4} M after 100 s does not significantly increase $[Ca^{2+}]_i$ further. The same response is seen with the addition of ATP 10^{-4} M after initial incubation with UTP 10^{-4} M. (e) PLC inhibitor U73122 (5×10^{-5} M) inhibits the biphasic increase in $[Ca^{2+}]_i$ in PC-3 cells induced by ATP 10^{-4} M. The inactive form of the PLC inhibitor U73343 (5×10^{-5} M) had no effect on the ATP response. All points are the mean (SEM) unless occluded by the symbol.

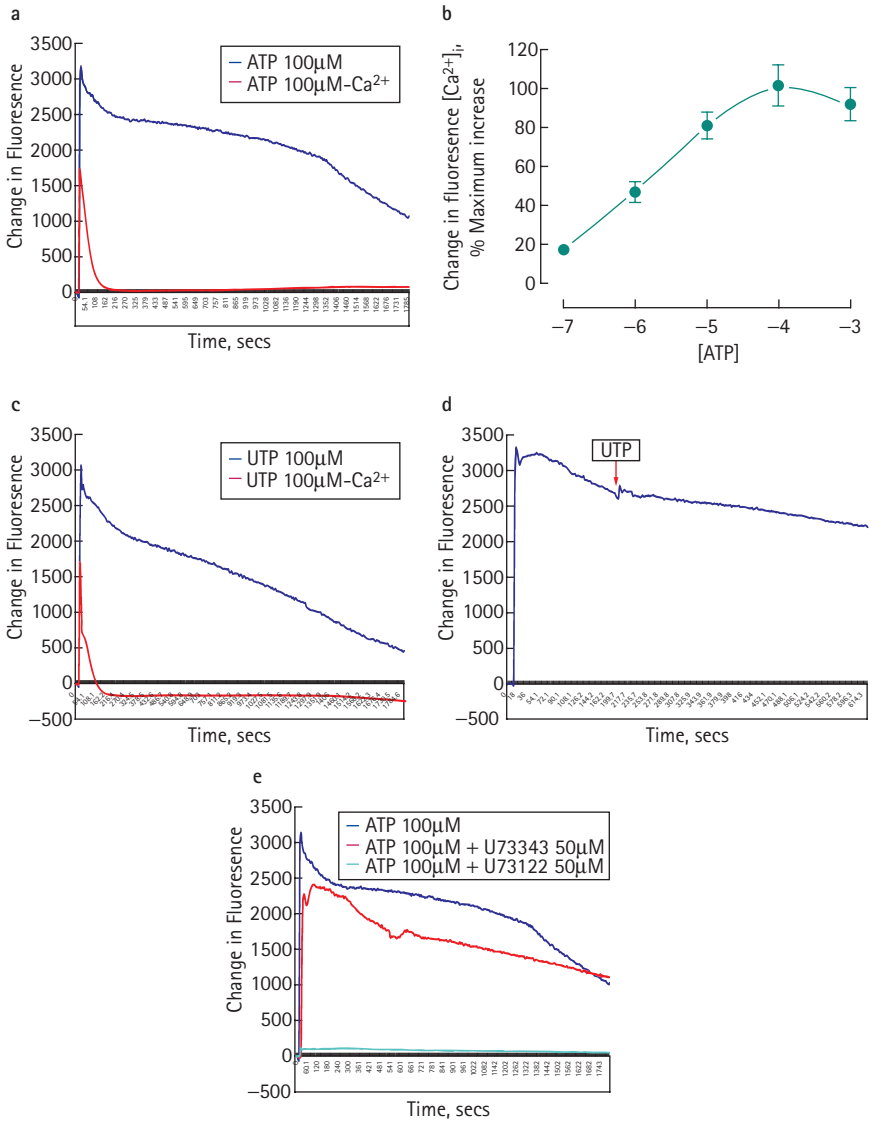
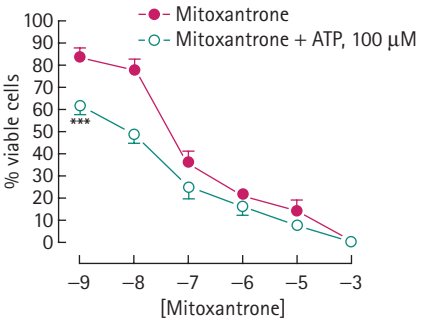


*FIG. 6. The effect of combining mitoxantrone (10^{-9}–10^{-4} M) and ATP (10^{-4} M) on the viability of PC-3 cells in vitro. All points are the mean (SEM) unless occluded by the symbol. ***P < 0.001.*



using the currently available agonists/antagonists it is difficult to define the role of the $P2Y_{11}$ receptor in the observed cytotoxic response.

Despite the similar mRNA expression, the normal prostate and HRPC cells differed considerably in their response to cell growth. PNT-2 cells were significantly less sensitive to the cytotoxic effect of ATP, and more responsive to the mitogenic effects of UTP. The order of agonist potency also differed from HRPC cells, raising the possibility that the control of growth in normal and cancerous prostate cells is different. This might either be due to the functional involvement of a different receptor complement, or an altered downstream response to the stimulation of the same receptor subtypes. Selective targeting of this aberrant pathway in the hormone-refractory cells would allow for the development of a novel therapeutic agent.

Although P2Y receptors have been shown to play an important role in mediating the growth response of ATP in other cell types, we found no role in the antineoplastic response in either prostate cancer cell line. P2X receptors are largely viewed as mediators of short-term, fast cell–cell communication. However, recent studies suggest that P2X receptors could also mediate trophic effects as shown by $P2X_7$ receptor-mediated apoptosis and the potential differentiating role for $P2X_5$ receptors [12,13]. Ryten *et al.* [14] showed that the activation of $P2X_5$ receptors mediated the stimulation of cell differentiation markers and thereby inhibited proliferation in skeletal muscle cells. The present study has identified the presence of functionally active $P2X_5$ receptors, which might inhibit the growth of HRPC cells via a combination of apoptosis induction and increased cellular differentiation resulting in reduction of proliferation. Assessing differentiation using cellular markers would help define the contribution of this process to the overall reduction in viable cell number.

The $P2X_7$ receptor has been shown to be an important mediator of apoptosis in various cell types [12]. The two HRPC cell lines differed in their expression of this receptor, with the DU-145 cell line showing no mRNA for this receptor subtype. Despite the presence of $P2X_7$ mRNA in PC-3 cells, a functional role for this receptor was not shown with respect to the observed cell death. The selective $P2X_7$ receptor antagonist, KN-62, had no effect on the ATP-mediated growth

inhibition, making the involvement of this receptor subtype unlikely, although the lack of effect of KN-62 at human $P2X_7$ receptors has been documented [15]. However, the lack of diffuse cellular uptake of LY after incubation with ATP also implies that the $P2X_7$ receptor was not initiating pore formation in these HRPC cells.

Recent studies have shown a wide variation in $P2X_7$ receptor function between samples, explained in part by four loss-of-function polymorphisms as well as rare mutations [16]. Despite our inability to show direct involvement of the $P2X_7$ receptor in the present study, its overall contribution to the growth response is difficult to exclude, as the underlying mechanism(s) of action of this receptor remain largely unknown.

ATP has been shown to inhibit the proliferation of PC-3 cells *in vitro*, although this was noted only after 4 days treatment [7]. Using the annexin V/PI early apoptosis detection kit, we were able to quantify ATP-induced apoptosis. There was a significant 2.5-fold increase in apoptosis after 72 h in the remaining adherent cells (annexin V-positive cells; control 1.9 (0.16)% vs ATP-treated 4.8 (0.17)%). This method of apoptosis detection is likely to give an underestimation of cell death due to the reduced adherence of dying cells, resulting in some apoptotic cells being lost during the staining procedure. All cells within the collected supernatant had evidence of either early or late apoptotic cell death.

In the calcium signalling (FLIPR) studies, ATP and UTP were equipotent and the inability of UTP to induce any additional increase in $[Ca^{2+}]_i$ after the addition of ATP confirmed that both agonists were working via the same pathway and mechanism. The ability of the PLC inhibitor U73122 (50 μM) to completely inhibit the increase in $[Ca^{2+}]_i$ further confirmed that ATP was acting via a G protein-coupled receptor (i.e. $P2Y_2$), with activation of PLC and inositol trisphosphate resulting in release of endoplasmic reticulum Ca^{2+}. The biphasic nature of the $[Ca^{2+}]_i$ response, with subsequent reduction in primary response and secondary sustained $[Ca^{2+}]_i$ after removal of extracellular Ca^{2+} ions, confirmed the secondary activation of store-operated channels, and resultant capacitative calcium entry of Ca^{2+} after depletion of endoplasmic reticulum Ca^{2+} stores. These responses are almost identical to those reported by Maaser *et al.* [9] in human oesophageal cancer cells, and in keeping with the early experiments described by Fang *et al.* [6] and Janssens and Boeynaems [7] in HRPC cells.

That both ATP and UTP had a similar effect on $[Ca^{2+}]_i$, while both were shown to have markedly opposite effects on cell growth (UTP increases viable cell number vs ATP that induces cell death) adds doubt to the role of the observed increases in $[Ca^{2+}]_i$ in ATP-induced growth inhibition. Studies by Vanoverberghe *et al.* [17] investigated the mechanisms of ATP-induced calcium signalling and growth arrest in DU-145 cells. Their studies confirmed the present findings of sustained increases in $[Ca^{2+}]_i$ after the addition of ATP, with evidence of endoplasmic reticulum Ca^{2+} store release and subsequent capacitative calcium entry. In addition, they also found that varying the concentrations of extracellular Ca^{2+} in the culture media had no significant effect on ATP-induced growth inhibition. Purinergic receptor stimulation by ATP might involve more than one secondary messenger system in addition, and possibly independent, to its $P2Y_2$ receptor-mediated effects on endoplasmic reticulum Ca^{2+} stores, which could potentially explain why ATP and UTP have such differing effects on cell growth, but such similar effects on $[Ca^{2+}]_i$.

In the final aspect of this study, we assessed the use of ATP in combination with chemotherapy. The aim of combined chemotherapy is to maximize antineoplastic activity while minimizing toxic side-effects of treatment, best achieved by combining drugs that have different mechanisms of action with an additive or synergistic effect and with different patterns of resistance to minimize cross-resistance. Increasing the dose of cell cycle nonspecific drugs such as mitoxantrone leads to increased cytotoxic effects but at the expense of more toxic side-effects.

The combination of ATP, at doses lower than its IC_{50}, with mitoxantrone, significantly increased the effect on cell death, effectively reducing the concentration of mitoxantrone needed to produce the same cytotoxic effect by a factor of 10. Assessment of the combined therapy using Bliss synergism showed that ATP worked in an additive rather than synergistic manner. Despite this, the use of ATP-mitoxantrone combination would allow either more cell death for the same chemotherapy drug concentration, or allow the same amount of cell death to be achieved at lower doses of chemotherapy, thereby reducing the concentration-dependent toxic side-effect of such drugs. While ATP combined therapy might have an important role to play in the future management of urological malignancies, more work is required to ascertain the best combination of agents and their optimum doses.

In summary, the present study has furthered our understanding of purinergic signalling in HRPC, suggesting that the antineoplastic action of ATP is likely to be mediated by $P2X_5$, and/or $P2Y_{11}$ receptors in DU-145 cells. The absence of $P2Y_{11}$ receptor mRNA in PC-3 cells makes the $P2X_5$ receptor the most likely receptor involved. Further pharmacological characterization is difficult at present due to the paucity of effective selective agonists and antagonists for these receptor subtypes. The observation of a selective change to the growth control of cancerous vs normal prostatic cells provides a potential therapeutic target in the treatment of this advanced urological malignancy.

CONFLICT OF INTEREST

None declared.

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Abbreviations: **HRPC**, hormone-refractory prostate cancer; **$[Ca^{2+}]_i$**, intracellular calcium ion concentration; **RT**, reverse-transcription; **FCS**, fetal calf serum; **MEM**, minimum essential medium; **bp**, base pairs; **8-SPT**, 8-thiophenyltheophylline; **PPADS**, pyridoxal-5′-phosphate-6-azophenyl-2′,4′ disulphonic acid; **KN-62**, (1-[N, O-bis (5-isoquinolinesulphonyl)-N-methyl-L-tryosyl]-4-phenylpiperazine; **ATPγS**, adenosine 5′-O-(3 thiotriphosphate; **BzATP**, benzoyl benzoyl ATP; **2-MeSATP**, 2-methylthio ATP; **2-MeSADP**, 2-methylthio ADP; **UTP**, uridine 5′-triphosphate; **UDP**, uridine diphosphate; **α,β-meATP**, α,β-methylene ATP; **LY**, lucifer yellow; **PI**, propidium iodide; **FLIPR**, fluorometric imaging plate reader; **HBSS**, Hanks' balanced salt solution; **CF**, cytotoxic fractions; **PLC**, phospholipase C.