Review Article

Purinergic receptor-mediated effects of adenosine 5′-triphosphate in urological malignant diseases

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Abstract: Adenosine 5′-triphosphate (ATP) mediates a variety of biological functions and has been shown to play a physiological role in almost every system in the body. In the genito-urinary system, extracellular ATP has been shown to play a functional role in several different capacities, ranging from nociception in the ureter and bladder, to erectile dysfunction via its action on different 'purinergic receptors'. Discovery of the trophic effects of ATP has led to a surge in interest in this signalling system in various malignancies. To date five P2 receptor subtypes have been implicated in the growth inhibition of cancer cells, namely $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$ and $P2Y_{11}$. Limited data are available on urological malignancies. ATP induces its anti-neoplastic effect primarily via purinergic receptor-mediated apoptosis via calcium-independent pathways, and this has been confirmed *in vitro* and *in vivo*. Studies have highlighted functional roles for the $P2X_5$ and/or $P2Y_{11}$ receptors in both hormone refractory prostate cancer and high-grade bladder cancer, although the contributory effect of pro-apoptotic $P2X_7$ receptors remains unclear. Clinical trials have shown intravenous ATP successfully attenuates a range of systemic symptoms associated with advanced malignancies. This raises the possibility that selective targeting of specific aberrant pathways may allow for treatment of advanced primary malignancies and their systemic effects.

Key words: Adenosine 5′-triphosphate, bladder, cancer, prostrate, purinergic.

Introduction

Prostate cancer is the most common malignancy to affect men in the western world. Radical treatment of early localized disease with surgery and radiotherapy has proven to be effective in the long term. The primary treatment of advanced metastatic prostate cancer with androgen ablation is rapid and effective at reducing symptoms, and although not curative, reduces the sequelae of local tumor growth and metastatic spread. Approximately 20% of patients fail to respond to first line hormonal therapy[1] and even those who do respond eventually develop hormone refractory disease. Although the time taken for this to develop may vary from patient to patient, once prostate cancer does become hormone refractory, it is difficult to treat and is usually fatal within 9–12 months.[2]

The search for alternative therapies for prostate cancer was spawned by the disappointing results of earlier trials of traditional anti-proliferative chemotherapy with overall response rates of only 8.7%, and no overall improvement in survival.[3] This poor response was attributable to the low cellular proliferation rate in metastatic prostate cancer. Any successful anti-neoplastic agent would need to have an apoptotic action in addition to any anti-proliferative effect.[4] So began the search for an alternative treatment for hormone refractory prostate cancer (HRPC).

Adenosine 5′-triphosphate (ATP) mediates a variety of biological functions including synaptic neurotransmission, smooth muscle contraction, and endocrine secretion, and has been shown to play a physiological role in almost every system in the body.[5,6] In the genito-urinary system, extracellular ATP has been shown to play a functional role in several different capacities, ranging from nociception in the ureter and bladder urothelium to erectile dysfunction via its action on different 'purinergic receptors'.[7,8] The discovery of the trophic effects of ATP on a variety of cells including induction of cell proliferation, differentiation, migration and death[5,9] led a surge in interest of the signaling system in the various malignancies. The first study of its effect in urological cancer was in HRPC[10] and demonstration of a primary apoptotic action in addition to anti-proliferative effects raised interest in the anti-neoplastic action of ATP in advanced urological tumors. Subsequent studies have also investigated the effect of ATP in bladder cancer. Here we review the current status of purinergic signaling in urological malignancies.

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A brief history of purinergic signaling

ATP was first discovered by the German chemist Karl Lohmann in 1929. Six years later, the Russian scientist Vladimir Engelhart first noted that muscle contractions required ATP. It wasn't until 1941 that Lipmann and Kalckar first discovered the central role of ATP in intracellular energy metabolism. Since then, the notion of ATP as Nature's 'universal energy store' has become widely accepted.

An extracellular role for purines was first demonstrated in 1929 by Drury and Szent-Györgyi,[11] who described the potent actions of purines on the mammalian heart. In 1959, Holton[12] first suggested that ATP may act as a neuronal messenger during antidromic stimulation of sensory nerve collaterals inducing alterations in vascular tone. However, it wasn't until 1970 that Burnstock and colleagues first proposed ATP as a non-adrenergic, non-cholinergic (NANC) neurotransmitter in the gut.[13] The discovery of neuronally released ATP during NANC parasympathetic neurotransmission in the bladder was made 2 years later.[14] By 1976, ATP was shown to be a co-transmitter released from both parasympathetic and sympathetic nerves.[15] The term 'purinergic signaling' was coined to describe this messenger system[16,17] and although it was initially received with much skepticism, the role of ATP as an extracellular neurotransmitter has now become universally accepted.[8] Acting via specific purinergic receptors, ATP has

subsequently been shown to be involved in numerous short-term and long-term cellular events, ranging from smooth muscle contraction and nociception to cell proliferation, cell differentiation and cell death.[9] Consequently, our need to understand this relatively recently uncovered messenger system in health and disease has become an important issue.

Purinergic receptors

Cell membrane bound purinergic receptors are subdivided into P1 receptors, where adenosine is the principal ligand, and P2 receptors, where the principal ligands are the purines adenosine diphosphate and ATP.[18] Four different G protein-coupled P1 receptor subtypes have been cloned: A_1, A_{2A}, A_{2B} and A_3.[19] P2 receptors are subdivided into two distinct families based on their molecular structure, transduction mechanisms and pharmacological properties.[20,21] P2X receptors are ligand-gated ion channels. Activation of P2X receptors results in rapid (within 10 ms) and selective permeability to cations (Na^+, K^+ and Ca^{2+}).[22] P2X receptors are mediators of fast excitatory neurotransmission in both the central and peripheral nervous systems, but are also widely expressed on non-neuronal cells.[5] P2Y receptors are G protein-coupled receptors. Activation of P2Y receptors results in a slower onset response (>100 ms) with coupling of G proteins leading to activation of different second-messenger systems including phospholipase C and inositol triphosphate (IP_3).[23]

Currently there are seven P2X receptors ($P2X_{1–7}$) and eight P2Y receptor subtypes ($P2Y_{1,2,4,6,11,12,13 \text{ and } 14}$) recognized in mammalian cells.[19,24] Each P2 receptor subtype has a different affinity for the various purinergic receptor agonists and antagonists. There are a growing number of selective antagonists to help establish functional assessment of each receptor subtype. Functional receptor characterization also depends on the assessment of relative orders of agonist potency. The development of newer, selective agonists and antagonists in this field will further our understanding of this emergent signaling pathway.

Cell growth and ATP

Factors influencing growth include the relative rates of proliferation, apoptosis and cellular differentiation altering cells ability to continue in the cell cycle. The mitogenic effect of adenine nucleotides was first highlighted by Gregory and Kern[25] in mouse thymocytes. Since then studies have shown that this mitogenic effect is primarily mediated by P2Y receptor subtypes and therefore likely involves activation of secondary messenger systems causing a cascade of events, resulting in cell proliferation.[9,26]

The cytotoxic effect of extracellular ATP has been demonstrated in numerous studies using different cell types, including rat hepatocytes[27] and mouse macrophages.[28] In the vast majority of cases, cell death was shown to be mediated via ATP-induced increased cell permeability to Ca^{2+} ions. This cytotoxic effect was later largely ascribed to the effect of extracellular ATP at the 'cytotoxic' pore-forming $P2X_7$ receptor subtype[29,30] inducing either necrotic or apoptotic cell death.[31]

Recent studies have also highlighted a role for purinergic receptors in cellular differentiation. The identification of $P2X_5$ receptors in the proliferating and differentiating cell layers of various rat stratified squamous epithelial tissues such as the cornea, tongue, soft palate, oesophagus and vagina, as well as in growing hair follicles, raised the possibility of a physiological role for these receptors in the turnover of continuously regenerating cells.[32] Further studies by Ryten *et al.*[33] demonstrated a functional role for $P2X_5$ receptors in the differentiation of mammalian skeletal muscle cells. Whereas previous understanding of P2X receptors focused on the control of short-term cellular responses, newer studies such as these highlighted a new and evolving role of P2X receptors in deciding the fate of cells within the cell cycle.

The cellular response to extracellular ATP, which has this ability to promote, differentiate and inhibit cell growth, largely depends on the cell type and the presence of specific functional purinergic receptors subtypes. The nature in which extracellular ATP is given may also be important in determining the cellular response. Di Virgilio[31] hypothesized that transient exposure to low concentrations of ATP may induce cell growth, while sustained exposure to higher concentrations would lead to cell death. Elucidation of the exact mechanism responsible for the differential effect of ATP would have important implications on the use of ATP, especially in cancer therapy. The discovery of selective expression of pro-apoptotic or anti-proliferative purinergic receptors in cancer cells would also allow for therapeutic targeting of tumors using adenine nucleotides.

Effect of ATP on human tumor cell growth

The anti-neoplastic activity of ATP was first shown by Rapaport in 1983,[34] who demonstrated that the addition of exogenous ATP to pancreatic and colon cancer cells inhibited cell growth by causing cell cycle arrest in the S-phase. Subsequent studies have shown an anti-neoplastic action of extracellular nucleotides in colorectal cancer,[35] leukaemia,[36] oesophageal cancer,[37] squamous cell skin cancer,[38] lung cancer,[39] cervical cancer[40] and melanoma.[41]

Different P2 purinergic receptor subtypes are involved in the 'growth inhibitory' response observed in the different malignant cell types. The anti-neoplastic action is either due to an inhibition of cell proliferation, the promotion of cell differentiation (resulting in inhibition of cell proliferation) and cell death, or a combination of these three processes.

To date, five P2 receptor subtypes have primarily been implicated in the growth inhibition of cancer cells, namely $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$ and $P2Y_{11}$,[42] with differing cell lines responding to receptor stimulation in different ways (see Fig. 1 and Table 1). $P2Y_1$ receptors decrease cell proliferation in melanoma[41] and squamous cell skin cancer.[38] In human esophageal and colorectal cancer cells, $P2Y_2$ receptor stimulation results in apoptotic cell death,[35,37] while in melanoma, stimulation of the same receptor increases cell proliferation.[41] The explanation for these divergent responses remains unclear at present.

In human leukemic cell lines, P2X receptor-mediated events result in growth inhibition.[36] $P2X_7$ receptors induce apoptosis in melanoma,[41] squamous cell skin cancer,[38] lung cancer[39] and cervical cancer.[40] Although the $P2X_7$ receptor is most widely accepted as the purinergic receptor mediator of apoptotic cell death, '$P2X_7$-independent' apoptosis has been described in fibroblasts[43] and murine tumor cells.[44] Exactly which P2X receptor subtypes were involved in this phenomenon remains unclear, although the cell differentiating effects of $P2Y_{11}$ receptors in leukemia cells[45] and $P2X_5$ receptors in skeletal muscle cells[33] and keratinocytes[38] may induce alterations to normal cell cycle progression and promote cell death.

ATP and urological malignancies

The role of purinergic receptors has only been assessed in two urological cancers to date: HRPC and bladder cancer. Although prostate cancer is the most common malignancy to affect men in the western world, bladder cancer is the second most common malignancy affecting the genitourinary tract.[46] These two malignancies often occur in the same patient. Chun[47] found that the rate of bladder cancer in patients with

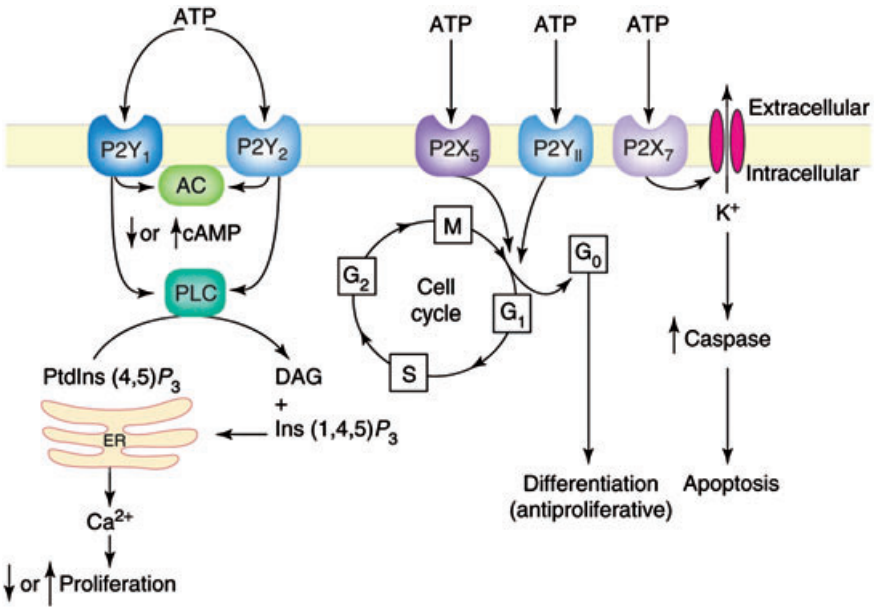

Fig. 1 Different mechanisms by which P2 receptor subtypes might alter cancer cell function. $P2Y_1$ and $P2Y_2$ receptors might affect the rate of cell proliferation by modulating adenylyl cyclase (AC) and altering the intracellular levels of cAMP, or by increasing the intracellular level of Ca^{2+} through the phospholipase C (PLC) pathway. $P2X_5$ and $P2Y_{11}$ receptor activation might switch the cell cycle from proliferation into a state of differentiation. The $P2X_7$ receptor activates the apoptotic caspase enzyme system. DAG, diacylglycerol; $Ins(1,4,5)P_3$, inositol (1,4,5)-trisphosphate; $PtdIns(4,5)P_2$, phosphatidylinositol (4,5)-bisphosphate. (Reproduced from White and Burnstock 2006[42] with permission from Elsevier.)

Table 1 Order of agonist potency and functional antagonists for the key anti-tumor P2 receptors

Receptor subtype	Agonists	Antagonists
$P2X_5$	ATP=2-MeSATP=ATPγS	Suramin, PPADS (non-selective)
$P2X_7$	BzATP>2-MeSATP≥ATP	KN62, O-ATP, Coomassie BBG, RN-6189, A-740003, A-438079, A-804598
$P2Y_1$	MRS2365>2-MeSADP=ADPβS	MRS2179, MRS2500, MRS2279
$P2Y_2$	2-thio-UTP>UTP=ATP	AR-C126313, PSB-716
$P2Y_{11}$	AR-C67085MX>BzATP=ATPγS	NF157, NF340

Compiled from Burnstock 2007.[8] ATP, adenosine 5′-triphosphate; UTP, uridine 5′-triphosphate.

prostate cancer is 18 times higher and the rate of prostate cancer in those with bladder cancer is 19 times higher than expected.

Approximately 90% of all bladder cancers in the western world are transitional cell carcinomas (TCC). Superficial bladder cancers, when the tumor is confined to the mucosa (stage Ta–Tis) or submucosa (stage T1), make up 75–85% of all bladder tumours.[48] Although low grade (G1) tumors are at low-risk of progression (10–20%) with a 98% 10-year survival, high-grade (G3) disease has a significantly higher risk of progression (33–67%) and a significantly reduced 10-year survival (35%).[49] Recurrence of high-risk disease after initial treatment with intravesical Bacillus Calmette-Guerin necessitates a radical surgical approach in those fit for surgery. Patients not medically fit for major surgery have limited options and a considerably higher mortality. Here, we review the effect of extracellular ATP on these two aggressive urological malignancies.

Prostate cancer

Fang *et al.*[10] first demonstrated that ATP could inhibit the growth of commercially available human HRPC cells (PC-3) *in vitro*. Using pharmacological characterization with various extracellular nucleotides, they concluded that this response was likely to be mediated by P2 receptors. Further studies by Janssens and Boeynaems[50] found that the ATP-induced growth inhibition in HRPC cell lines PC-3 and DU145 was largely due to activation of P2X receptor subtypes, and primarily involved induction of apoptosis with some evidence of a delayed anti-proliferative effect. However, the exact complement of receptor subtypes involved and their mechanism of action remained unclear.

Studies from our own laboratories compared HRPC cell lines (PC-3 and DU145) with commercially available normal prostate cells (PNT-2).[51] 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 (19 ± 3.2% *vs* 45 ± 2.3%, after ATP 0.1mM), and more responsive to the mitogenic effects of uridine 5′-triphosphate (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 may be due either to the functional involvement of a different receptor complement, or an altered downstream response to the stimulation of the same receptor subtypes. Pharmacological characterization suggested that the anti-neoplastic action of ATP was likely to be mediated by $P2X_5$ and/or $P2Y_{11}$ receptors in DU145 cells. The absence of $P2Y_{11}$ receptor mRNA in PC-3 cells made the $P2X_5$ receptor the most likely receptor involved in this cell line.

The discovery of $P2X_7$ receptor mRNA in PC-3 cells raised hopes of a pivotal role for this pro-apoptotic receptor in the observed cell death. However, functional studies using the selective antagonist KN-62, and assessment of $P2X_7$ receptor-mediated cell membrane pore formation

(using lucifer yellow stain) failed to demonstrate a functional role for these receptors. Coupled with the presence of $P2X_7$ receptor mRNA in the normal PNT-2 cells and its absence in DU145 cells, which despite the absence of this receptor had a similar cytotoxic response to ATP as PC-3 cells, left the explanation of the exact functional role of this receptor subtype uncertain.

The lack of effect of KN-62 at a human $P2X_7$ receptor has been reported previously, where it failed to block permeability lesions to Ca^{2+} and Ba^{2+} and subsequent cytotoxic pore formation.[52] There are known to be many polymorphisms of the $P2X_7$ receptor,[53] which, in addition to conferring a loss of function, may alter the activity of the receptor. Another possibility could be the activation of alternative downstream events. Wang *et al.*[40] found that ATP stimulated apoptosis via $P2X_7$ receptor mediated caspase-9-mitochondrial pathways in cervical carcinoma cells.

There is a differential expression of $P2X_7$ receptors in patients with normal prostates compared with those with prostate cancer. Slater *et al.*[54] found expression of non-functional $P2X_7$ cytolytic purinergic receptors in all 116 pathology specimens of prostate cancer, irrespective of Gleason grade or patient age. $P2X_7$ receptors were also found in normal epithelial cells adjacent to tumor margins, but not in normal tissues from patients with no evidence of cancer, raising the possibility of the appearance of such receptors as an early marker of prostate cancer. What functional role this may play in the development or treatment of prostate cancer is unclear, and the exact underlying mechanism of action of this receptor remains largely unknown.

The exact control of growth in HRPC is unclear. In hormone-sensitive normal prostate and prostate cancer cells, androgen ablation leads to apoptotic cell death. In these cells androgen ablation leads to a sustained rise in intracellular calcium ion concentration ($[Ca^{2+}]_i$), leading ultimately to programmed cell death.[55] This response to androgen ablation is lost in hormone refractory cells. Studies by Martikainen *et al.*[56] showed that modest elevations in $[Ca^{2+}]_i$ for sufficient time, achieved using calcium ionophores such as ionomycin, induced apoptotic cell death in HRPC cells, raising the possibility that alterations in calcium homeostasis could still be the key to apoptosis induction in HRPC.

ATP has been shown to increase $[Ca^{2+}]_i$ in various human cancer cell lines *in vitro*, including prostate cancer[10,51] and this has been proposed as a possible mechanism for ATP-induced cell death. ATP induces a biphasic increase in $[Ca^{2+}]_i$, with an immediate release of endoplasmic reticulum (ER) stored Ca^{2+}, and secondary activation of store-operated channels, with resultant capacitative calcium entry of Ca^{2+} after depletion of ER Ca^{2+} stores. We found ATP and UTP to be equipotent at increasing $[Ca^{2+}]_i$ in HRPC cells, while both were shown to have markedly opposite effects on cell growth (UTP increases viable cell number whereas ATP induces cell death).[51] Complete blockade of $[Ca^{2+}]_i$ increase was observed after use of the phospholipase C inhibitor U73122, confirming the role of a G protein-coupled receptor (i.e. $P2Y_2$) in this response, contrary to the cytotoxic effects of ATP in HRPC cell growth. Studies by Vanoverberghe *et al.*[57] also confirmed this poor correlation between $[Ca^{2+}]_i$ and control of HRPC cell growth. They found that varying the concentrations of extracellular Ca^{2+} in culture media had no significant effect on ATP-induced growth inhibition, thereby denoting either an alternative mechanism, or secondary messenger, in ATP induced-apoptotic cell death.

Vanoverberghe *et al.*[57] hypothesized that decreases in the intracellular Ca^{2+} pool was more relevant to the observed cell death, backed up by experiments showing pretreatment with thapsigargin, at a level where it had no apoptotic effect itself (1 nM), prevented ATP-induced growth inhibition (100 μM) by decreasing the Ca^{2+} pool content. Interestingly, although both 1 nM thapsigargin and 100 μM ATP reduced Ca^{2+} pool content by a similar amount, only thapsigargin alone had no growth inhibitory effects.[57] As thapsigargin and ATP work on the ER in different ways to lower the Ca^{2+} pool (sarco/endoplasmic reticulum Ca^{2+} ATPase pump inhibitor *vs* IP_3 receptor activation), they concluded that the secondary mechanisms involved may be more important than the level of reduction in the intracellular Ca^{2+} pool alone.

One possibility could be potential alterations to the intracellular production and levels of Bcl-2 proteins by extracellular ATP. Overexpression of Bcl-2 proteins are associated with a prevention of apoptosis, and are a common finding in cancer cells. Miyake *et al.*[58] previously showed that the treatment of HT1376 bladder cancer cells with ionomycin induced apoptosis and decreased the mRNA and receptor expression of the anti-apoptotic Bcl-2 proteins, while increasing the expression of pro-apoptotic Bax proteins. At present, no studies have explored the effect of ATP on Bcl-2 expression in prostate cancer, or any other malignancy, and this would be an interesting avenue of future research.

The *in vitro* cytotoxic effects of extracellular ATP have also been confirmed *in vivo*. We found that daily intraperitoneal injections of ATP significantly reduced the effect of subcutaneously implanted DU145 and PC-3 cells in male nude athymic mice (57–69% reduction in the growth of freshly implanted or established DU145 and PC-3 cells respectively) (Fig. 2a,b).[59] No side effects or complications related to ATP treatment were seen throughout the experiment. Light and electron microscopy were used to confirm that the inoculated tumor cells retained their original phenotype and cellular characteristics.

Although these experiments validated the relevance of the *in vitro* experiments on the primary growth of HRPC, they gave no information about the effect of ATP on tumor metastases. An orthotopic model of prostate cancer would add to our understanding of this process and the potential effect of ATP. Prostate cancer primarily metastasizes to bones in the axial skeleton. Bisphosphonates, such as zolendrenic acid, licensed for use in the treatment of bone metastases in patients with HRPC, have previously been shown to inhibit prostate carcinoma cell adhesion to bone.[60] Bisphosphonates inhibit growth, attachment and invasion of cancer cells in culture and promote apoptosis. A recent study has shown that this is, in part, due to the formation of a novel ATP analog (ApppI) which is able to induce apoptosis.[61] Further assessment of this phenomenon, and its possible interaction with the functional $P2X_7$ receptors found on osteoclasts[62] may help further our understanding of ATP treatment and purinergic receptor pathways in prostate cancer.

Bladder cancer

The effect of ATP has been investigated in grade 3 (G3) TCC.[63] Commercially available HT-1376 cells were found to express the same purinergic receptor mRNA as PC-3 prostate cancer cells ($P2X_{4,5,7}$ and $P2Y_{1,2,4,6,11}$). ATP reduced cell growth in a concentration-dependent manner, via the induction of P2 receptor-mediated apoptosis. Pharmacological profiling implicated $P2X_5$ and/or $P2Y_{11}$ receptors in this anti-neoplastic response, although any possible contributory effect of $P2X_7$ receptors could not be discounted. This functional receptor profile, and the order of agonist potency was the same as that seen in HRPC cells, although G3 TCC cells were more sensitive to the cytotoxic effects of ATP (reduction of growth 88.5 ± 4.4% vs. 45 ± 2.3% for PC-3 cells at ATP 0.1mM). Our results suggest that the two most common advanced urological malignancies may have a common therapeutic purinergic target despite their differing cellular type and origin (transitional cells in the bladder *vs* prostate adenocarcinoma).

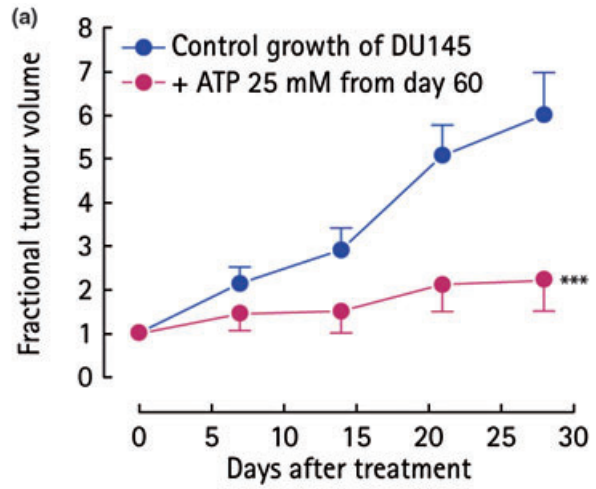

Fig. 2 (a) Effect of adenosine 5′-triphosphate (ATP) on the fractional growth of hormone refractory prostate cancer (HRPC) DU145 tumor cells *in vivo* after 60 days initial growth; and (b) Effect of ATP on the growth of implanted DU145 tumor cells *in vivo* after 60 days initial growth; the lower mouse received ATP treatment versus no treatment in the upper mouse. (Reproduced from Shabbir *et al.* 2008[59] with permission from Blackwell Publishing.)

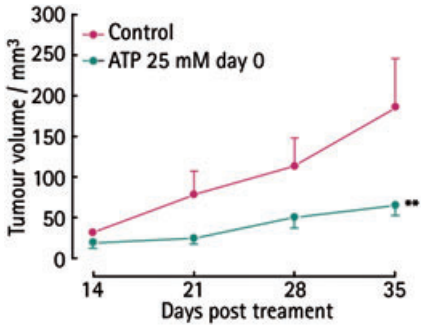

Fig. 3 Effect of daily intraperitoneal adenosine 5′-triphosphate (ATP) (25 mM) from day 0 on the growth of freshly implanted human bladder transitional cell carcinomas (TCC) HT-1376 tumor cells *in vivo*. (Reproduced from Shabbir *et al.* 2008[63] with permission from Blackwell Publishing.)

In our study, we found that ATP was able to significantly increase apoptosis after 72 h. Although studies have demonstrated a potential differentiating role for $P2X_5$[33,38] and $P2Y_{11}$ receptors,[45] no studies have implicated these receptors in the induction of apoptosis. Apoptosis has classically been linked to the $P2X_7$ receptor, although, despite the presence of $P2X_7$ receptor mRNA, we were unable to elicit a significant functional role for this receptor subtype. Ryten *et al.*[33] demonstrated that the activation of $P2X_5$ receptors mediated the stimulation of cell differentiation markers and thereby inhibited proliferation in skeletal muscle cells. It is therefore possible that activation of $P2X_5$ receptors in bladder cancer leads to cellular differentiation, resulting in cells unable to continue the cell cycle, which subsequently undergo apoptosis. This may explain the delay in apoptosis detection, with no significant increase noted after 24 h incubation with ATP. Assessment of cell differentiation using markers would help define the contribution of this process to the observed growth inhibition, and further clarify the anti-neoplastic mechanism of ATP in bladder cancer.

In vivo experiments mirrored the *in vitro* findings, with a reduction in mean implanted tumor volume by 64.3% after daily intraperitoneal treatment with ATP (Fig. 3). No obvious side effects relating to treatment were noted in any experimental group. Histological analysis of the neoplasms in control mice using hematoxylin and eosin staining and transmission electron microscopy (TEM) showed tumors maintained the classical characteristics of urinary TCCs. Although ATP-treated tumors were significantly smaller, light microscopy revealed no other histological changes. TEM detected an increase in both apoptotic bodies and necrosis in treated tumors.[63]

The distinct advantage with bladder tumors is that direct instillation of chemotherapeutic agents via a urinary catheter is easily achievable and allows drugs to be given at more concentrated levels locally to induce a sufficient response, while reducing systemic side effects. This may benefit the use of ATP either alone or in combination in future trials.

Future directions

Combination therapy

The primary principle of combination chemotherapy is to maximize anti-neoplastic activity while minimizing toxic side effects of treatment. This is best achieved by combining drugs, which have different mechanisms of action with an additive or synergistic effect and with different patterns of resistance to minimize cross-resistance. In bladder cancer, the combination of ATP with the established anti-tumor antibiotic mitomycin C (MMC) significantly increased its effect on cell death, reducing the chemotherapeutic drug concentration at which 50% of cells were killed by a factor of 10[63] (Fig. 4a). The same effect was seen with ATP and Mitoxantrone, an anti-tumor antibiotic approved for use in the treatment of HRPC[51] (Fig. 4b). However, the cytotoxic effect of these combinations were additive only and not synergistic. This is probably explained by the respective mechanisms of action. Both anti-tumor antibiotics are cell cycle non-specific, whereas ATP has previously been shown to induce checkpoint defects leading to S-phase arrest, preventing further progression in the cell cycle, and eventual apoptosis.[34] Cell cycle non-specific drugs work effectively on all cancer cells. With ATP thought to work primarily only in the S-phase, the decrease in the surviving fraction of cells after exposure to the chemotherapeutic drug would decrease the number of viable cells for ATP to induce its cytotoxic effect. With this in mind, ATP would be better in combination with a chemotherapeutic drug known to work in a

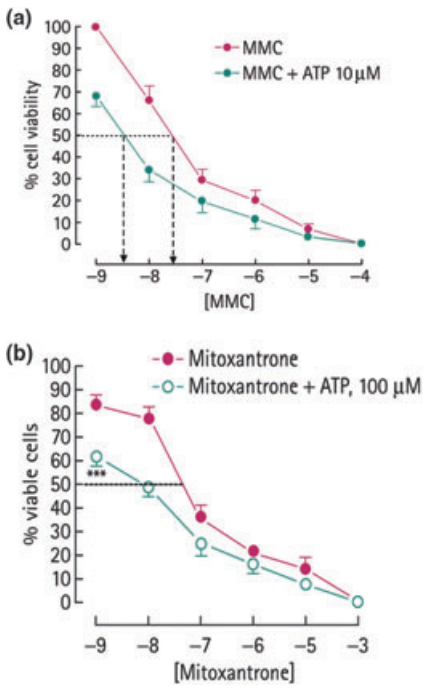

Fig. 4 (a) Dose–response curve of the effect of combining adenosine 5′-triphosphate (ATP) with mitomycin C (MMC) versus MMC alone on the viability of human bladder transitional cell carcinomas (TCC) HT1376 cells *in vitro*. (b) The effect of combining mitoxantrone and ATP on the viability of hormone refractory prostate cancer (HRPC) PC-3 cells *in vitro*. All points are the mean (SEM) unless occluded by the symbol. ***$P < 0.001$. (a reproduced from Shabbir *et al.* 2008[63] and b from reproduced from Shabbir *et al.* 2008[51] with permission from Blackwell Publishing.)

different phase of the cell cycle, to prevent any overlap, and to increase the chance of synergism. To this effect, the addition of docetaxol, active in the G2/M phase and on bcl-2 phosphorylation, would theoretically be more advantageous in combination with ATP.

Clinical trials

The ultimate goal of any laboratory-based medical research is to see translation of this work to treatment in patients with disease. Intravenous ATP has already been safely trialed in patients with lung cancer. Agteresch *et al.*[64] investigated the pharmacokinetics of intravenous ATP in 28 patients. Treatment was well tolerated with no side effects in two-thirds of the group. Side effects included chest tightness (15%) or dyspnoea (10%), which was mild (level 1 or 2 by the US National Cancer Institute Criteria) and transient, resolving within minutes of decreasing the infusion rate or stopping the infusion. Other minor side effects included flushing and nausea in 5%, light-headedness in 3%, headache and sweating in 2% and palpitations in 1%.

In keeping with murine models, ATP treatment has been shown to maintain body weight and decrease cancer cachexia in human studies.[65] In the murine cancer models, intraperitoneal ATP inhibited weight loss in the animals with advanced tumor growth independent of its primary anti-neoplastic action. This anti-cachectic effect was thought to occur primarily via the ATP breakdown product, adenosine, which had little anti-neoplastic activity, but was effective at reducing weight loss. However, the anti-cachectic effect of ATP was greater than that seen with adenosine alone, implying that some other mechanism must be involved, at least in part.[66] In their trial, Agteresch *et al.*[65] found intravenous ATP infusions maintained body weight, muscle strength, serum albumin concentrations and quality of life in cachectic patients with advanced lung cancer over the 6-month period of the investigation. In 2003, Agteresch *et al.*[67] also showed, in a randomized controlled trial, that ATP infusions in patients with advanced non-small cell lung cancer significantly increased overall survival (9.3 months ATP-treated *vs* 3.5 months for control), supporting the theory that ATP may treat the underlying malignancy as well as its systemic effects, although larger trials are needed to confirm this. A further trial is currently underway by the same group, investigating the effects of ATP treatment in combination with radiotherapy for non-small cell lung carcinoma. This multicentre, double-blind, randomized controlled trial will focus on the effects of ATP on survival, tumor response, nutritional status, and quality of life; first results will be available in 2010.[68]

While the exact mechanism for this response remains unclear, it does raise the possibility that ATP may be used for the treatment of both a primary tumor and the systemic side effects of the tumor in a patient with advance disease, as demonstrated in the murine *in vivo* models. This could potentially have a considerable effect on the management of patients with advanced urological malignancy.

Summary

As our understanding of purinergic signaling increases, so does the range of biological events found to be dependent on this messenger system both in health and disease. The discovery of purinergic receptor-mediated apoptotic pathways in advanced urological malignancies, irrespective of the cellular type or origin has raised the possibility of possible future therapies for these aggressive malignancies. Studies have shown functional roles for the $P2X_5$ and/or $P2Y_{11}$ receptors, although any possible contributory effect of $P2X_7$ receptors could not be discounted. Selective targeting of these aberrant pathways would allow for the development of a novel therapeutic agent that could not only treat the primary malignancy, but also improve the systemic symptoms associated with advanced malignancy.

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