

Purinergic receptor-mediated effects of ATP in high-grade bladder cancer

Majid Shabbir, Mina Ryten, Cecil Thompson*, Dimitri Mikhailidis* and Geoffrey Burnstock

Autonomic Neuroscience Centre, and Departments of *Surgery and Clinical Biochemistry, Royal Free and University College Medical School, London, UK.

Accepted for publication 29 June 2007

Study Type – Aetiology (case series)
Level of Evidence 4

OBJECTIVE

To assess whether the antineoplastic action of extracellular ATP seen in hormone-refractory prostate cancer extends to other aggressive urological malignancies by investigating its effect in high-grade bladder cancer cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

HT-1376 cells (human grade 3 transitional cell carcinoma) were incubated with various purinergic receptor agonists and antagonists and their effects on cell growth was examined

in vitro. The presence of different P2 receptor mRNAs was determined using reverse transcriptase-polymerase chain reaction. The effect of combining ATP with the cytotoxic agent mitomycin C (MMC) was also investigated. Models of tumour outgrowth in athymic mice were used to examine the effect of ATP on tumour growth *in vivo*.

RESULTS

HT-1376 cells expressed P2X_{4,5,7} and P2Y_{1,2,4,6,11} receptor mRNA. ATP significantly reduced cell growth in a concentration-dependent manner via the induction of P2 receptor-mediated apoptosis. Pharmacological profiling implicated P2X₅ and/or P2Y₁₁ receptors in this antineoplastic response, the same receptor subtypes shown to be active in prostate

adenocarcinoma, despite the differing cellular origin. ATP and MMC combined in an additive manner. Intraperitoneal injections of ATP significantly reduced the growth of implanted tumour cells by a combination of apoptosis and necrosis.

CONCLUSIONS

ATP effectively reduces the growth of high-grade bladder cancer cells *in vitro* and *in vivo*. This highlights the potential use of ATP in the treatment of advanced urological malignancies irrespective of the cellular origin.

KEYWORDS

ATP, bladder, mitomycin, P2 receptors, transitional cell carcinoma

INTRODUCTION

Bladder cancer is the second most common malignancy affecting the genitourinary tract. In the Western world ≈ 90% of all bladder cancers are TCCs that are confined to the bladder at presentation in ≈ 75% of cases.

High-grade (G3) superficial bladder cancer (SBC) has a greater risk of recurrence and progression [1]. Early recognition and treatment is important for the successful management of such 'high-risk' bladder cancer. Recurrence of high-risk disease on a re-staging TUR in a patient fit for surgery usually leads to an immediate radical cystectomy. For patients not medically fit for major surgery or those unwilling to undergo such a major procedure, the use of intravesical BCG is indicated. While intravesical BCG has been shown to effectively reduce the recurrence of high-risk SBC compared with standard intravesical MMC alone, it has no effect on disease

progression or survival [2] and has a greater incidence of localized and systemic side-effects compared with MMC [2].

There has been very little change in the management of high-risk SBC over recent times, and the need for radical cystectomy remains high. The use of fluoroscopic TUR has improved detection of SBC and carcinoma *in situ* in particular [3]. In addition, the active administration of MMC using electromotive drug administration has improved response rates vs more traditional passive instillation of MMC in high-risk SBC, achieving levels similar to intravesical BCG [4]. However, neither of these techniques are widely available.

The need for novel therapeutic approaches to treatment is crucial to the future management of high-risk bladder cancer, and bladder preservation is both essential and desirable, wherever possible, to clinicians and patients alike.

The extracellular signalling molecule ATP has been shown to mediate various biological functions including synaptic neurotransmission, nociception, smooth muscle contraction, and endocrine secretion [5]. ATP acts via specific P2 receptor subtypes, P2X ionotropic (P2X₁₋₇) and P2Y metabotropic (P2Y_{1,2,4,6,11,12,13,14}), and can induce trophic responses in various cells that include cell proliferation, cell differentiation, and cell death [6]. It has been shown that purinergic agonists are able to inhibit the growth of poorly differentiated hormone-refractory prostate adenocarcinoma cells *in vitro* via P2 receptor-mediated apoptosis [7]. However, no studies have investigated the effect of P2 receptor agonists on human TCC.

The aim of this study was to investigate the role of purinergic signalling in the growth of high-grade (G3) human bladder cancer *in vitro*, as a basis for investigating whether purinergic agonists might be used effectively as an antineoplastic agent to treat high-risk,

superficial TCC of the bladder. In addition, we investigated the effect of combining the cytotoxic effect of MMC with the apoptotic effect of ATP to determine if they interact in a synergistic or additive way to enhance their antineoplastic activity *in vitro*. Finally we assessed the effect of ATP on the growth of high-grade (G3) human bladder cancer *in vivo*, using established models of tumour outgrowth in immuno-compromised nude athymic mice, as previously described by Rapaport [8].

MATERIALS AND METHODS

IN VITRO EXPERIMENTS

The human bladder cancer cell line, HT-1376, was obtained from the European Collection of Cell Cultures (Salisbury, Wilts., UK). These cells were derived from a 58-year-old woman with high-grade (G3) TCC of the bladder who had not been treated with chemotherapy or radiotherapy. Studies have confirmed that HT-1376 cells maintain patterns of gene expression similar to the stage of progression of the tumour of origin, in common with other high-grade bladder cancer cell lines, thereby validating their use in research [9]. Cells were grown in minimum essential medium (MEM) with 2 mM L-glutamine supplemented with 10% fetal calf serum (Sigma Chemical Co., Poole, UK), 1% non-essential amino acid solution (Sigma) and a 1% antibiotic solution containing penicillin, streptomycin and amphotericin B (Sigma) in a humidified atmosphere at 37 °C containing 5% CO₂.

For reverse-transcriptase-PCR (RT-PCR) the total RNA was extracted from HT-1376 cells in culture using the SV Total RNA Isolation system (Promega, WI, USA). Reverse transcription and cDNA amplification for all the P2 receptors was carried out with a thermal cycler (Hybaid Ltd, UK) in a two-step protocol using Ready-To-Go RT-PCR Beads (Amersham Pharmacia biotech, UK). Each 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: P2X₁, 510 base pairs (bp); P2X₂, 355 bp; P2X₃, 695 bp; P2X₄, 295 bp; P2X₅, 595 bp; P2X₆, 520 bp; P2X₇, 674 bp; P2Y₁, 527 bp; P2Y₂, 637 bp; P2Y₄, 424 bp; P2Y₆, 364 bp; and P2Y₁₁, 410 bp. 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.

For assessment and pharmacological characterization of P2 receptor-mediated effects on cell growth HT-1376 cells were seeded at a density of 80 000 cells/mL in 24-well plates. After 24 h, the number of adherent viable cells in control wells was recorded using the haemocytometer method of counting Trypan blue excluding cells. ATP in increasing concentrations (10⁻⁷–10⁻³ M, *n* = 12) was added to the medium either as a single dose at 24 h or daily in fresh medium for 72 h to each different row of wells. After 72 h of initiation of treatment with ATP, cell viability was assessed in both control and treated wells. Experiments were repeated in the presence of the P1 receptor antagonist 8-thiophenyltheophylline (8-SPT; 10⁻⁶–10⁻⁴ M, *n* = 9), the non-specific P2 receptor antagonist suramin (10⁻⁶–10⁻⁴ M, *n* = 9) and the P2X₇ receptor antagonist 1-[N, O-bis (5-isoquinolinesulphonyl)-N-methyl-L-tryosyl]-4-phenylpiperazine (KN-62; 10⁻⁶–10⁻⁴ M, *n* = 9). 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), UTP, UDP and α,β-methylene ATP (α,β-meATP) in place of ATP (all 10⁻⁷–10⁻⁴ M, *n* = 9). All agonists and antagonists were obtained from Sigma.

To assess ATP-mediated apoptosis HT-1376 cells were grown on coverslips and incubated in complete MEM medium in the absence or presence of ATP (10⁻³ M, *n* = 5). After 72 h, any cells still adherent to the coverslips were analysed for apoptosis induction using an annexin V-fluorescein isothiocyanate cell-binding assay and propidium iodide (PI), stained according to the manufacturer's

protocol (Sigma). The supernatant containing cell fragments and dead cells was removed and analysed separately using the same method for apoptosis detection. Stained cells were viewed using a fluorescence microscope (Axioplan, Zeiss, Oberkochen, Germany). Supernatants and coverslips were mounted on slides with a 'blinded' code. The proportion of cells staining positive for annexin V and negative for PI (early apoptosis) were counted in three representative high-powered fields from each sample.

Cell permeabilization, by activation of the pro-apoptotic P2X₇ receptor, was assessed by observing the differential uptake of the fluorescent dye lucifer yellow (LY) in ATP-treated cells. HT-1376 cells were grown on coverslips and incubated in complete MEM medium containing LY at 5 mg/mL. Cells were incubated in the absence and presence of ATP (10⁻³ M) for 15, 30, 45 or 60 mins at 37 °C (*n* = 3 at each time point). At the end of the incubation period, both control and ATP-treated cells were rapidly washed four times with PBS and viewed immediately using a fluorescence microscope (Axioplan, Zeiss) with a dark field attached to a digital camera (Leica DC200, Heerbrugg, Switzerland). Cells within the supernatant, and those still adherent to the coverslips were mounted on slides with a 'blinded' code.

To assess the effect of ATP in combination with chemotherapy HT-1376 cells were seeded at a density of 80 000 cells/mL in 24-well multi-well plates. After 24 h, the number of viable cells in the control wells was recorded using the haemocytometer method, counting Trypan blue excluding cells. The chemotherapeutic drug MMC (Sigma) was then added to experimental wells at increasing concentrations (10⁻⁹ M–10⁻⁴ M, *n* = 12). After 72 h of incubation, cell viability was again assessed. Experiments were then repeated with cells incubated either with MMC alone, or with a combination of MMC and ATP (10⁻⁵ M, *n* = 12), at a concentration significantly below its IC₅₀ (3.8 × 10⁻⁵ M).

IN VIVO EXPERIMENTS

Animal models were developed under license from the Home Office (UK) and ethically approved by local committee. Male nude athymic mice (MF1-nude; 6–8 weeks old, Harlan UK Ltd, Bicester, UK) were used. Mice were maintained in a specific pathogen-free

TABLE 1 Well-being score assessment

Variable	Score
A. Appearance:	
Normal	0
Decreased grooming	1
Piloerection	2
Hunched posture	3
B. % Loss of body weight: (compared with age-matched control)	
0–4	0
5–9	1
10–14	2
15–20	3
C. Provoked behaviour:	
Normal	0
Agitated	1
Isolated	2
Lethargic	3
D1. Clinical signs for s.c. tumour model:	
Tumour size, cm	
<0.5	0
0.5–1.0	1
1.5–2.0	2
>2.0	3
Any evidence of ulceration over tumour	4
D2. Clinical signs for orthotopic model:	
Difficulty in breathing	4
Urinary retention	4
Abdominal distension	4

For sections A–C, consecutive readings of maximum score (3) add a score of +1 per section. Overall score = sum of scores from sections A, B, C and D: maximum score 16. Strategies (according to final overall score):

- 0–4 Normal animal
- 5–8 Increased monitoring. Analgesia as required.
- 9–12 Increased monitoring. Analgesia as required. If animal is in treatment group, all therapy to be stopped. Involve and seek advice of named Veterinary Surgeon.
- 3–16 Kill animal by method outlined in schedule 1 (UK Home Office Regulations) without delay.

environment, housed in individually ventilated cages (maximum five per cage), and given sterilized food and water *ad libitum*. All procedures were performed aseptically in a pathogen-free environment. All mice were weighed at the start of the experiment and weekly thereafter, and assessed weekly for adverse events and well-being assessment scores were completed (see Table 1).

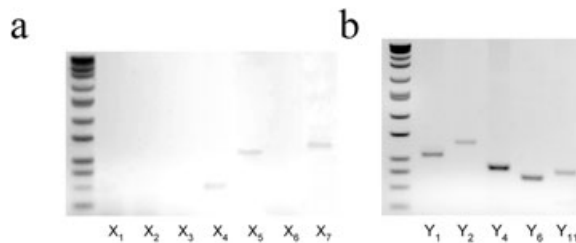


FIG. 1. Expression of P2X and P2Y receptor mRNA in HT-1376 bladder cancer cells using RT-PCR. (a) P2X_{4,5,7} receptor mRNA and (b) P2Y_{1,2,4,6,11} receptor mRNA.

Tumours were induced by s.c. inoculation of 1×10^6 HT-1376 cells (>90% viability) suspended in 75 μ L appropriate complete MEM and 75 μ L Matrigel (ratio 1 : 1, total inoculation volume 150 μ L). Matrigel, an extract of basement membrane proteins, induces rapid tumour development after s.c. injection and improves the growth of human tumours in athymic mice. Cells were mixed with Matrigel and whirl mixed before injection into the right flank using a 20-G needle while the mice were lightly anaesthetized with halothane.

Experiment 1: To assess the effect of ATP treatment on freshly implanted tumours by administering daily i.p. injections of ATP 25 mM in 1 mL sterile saline, pH adjusted to 6.2 using NaOH (safe dose based on previous studies using ATP *in vivo* [8]) starting the day after tumour inoculation. A control group received an equivalent volume of the vehicle (sterile saline) daily IP (five mice in each group).

Experiment 2: To assess the change in the rate of growth of an established tumour after ATP treatment. The same therapeutic regimen was used as before, but treatment was commenced once the tumour growth was well established after 14 days (five mice in each group).

To evaluate treatment tumour size was measured weekly using standard callipers. Tumour volume was calculated for experiment 1 using the standard formula:

$$\text{tumour volume} = \text{length} \times \text{width} \times \text{height} \times 0.52$$

The rate of change of established tumour growth for experiment 2 was calculated using the formula:

$$\text{fractional tumour volume} = \frac{(\text{volume on day measured})}{(\text{initial pretreatment tumour volume})}$$

At the end of the experiments, all mice were killed using an increasing concentration of

CO₂ in accordance with Schedule 1 of the Home Office (UK) guidelines. Tumours were removed and examined histologically either using light microscopy (tissue fixed in 10% neutral buffered formalin, paraffin wax-embedded and stained with haematoxylin and eosin (H&E)) or transmission electron microscopy (TEM; fixed in 1.5% glutaraldehyde and post-fixed using osmium tetroxide). Histological review of the pathology slides was done in conjunction with a local histopathologist with a specialist interest in uro-oncology.

All data are expressed as the mean (SEM). Cumulative response curves were plotted and compared by means of the software Prism 3.0 (GraphPad Software, Inc., San Diego, CA, USA) using a two-way ANOVA followed by a *post hoc* Bonferroni's test. The significance of ATP-induced apoptosis was assessed using a two-tail Student's *t*-test. Assessment of synergy between ATP and MMC was done using the calculation for 'Bliss Synergy' according to the fractional product analysis [10]. 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 approximately equal to 1 indicated additivity, and values < 1 indicated Bliss antagonism. For all analyses *P* < 0.05 was considered to indicate statistical significance.

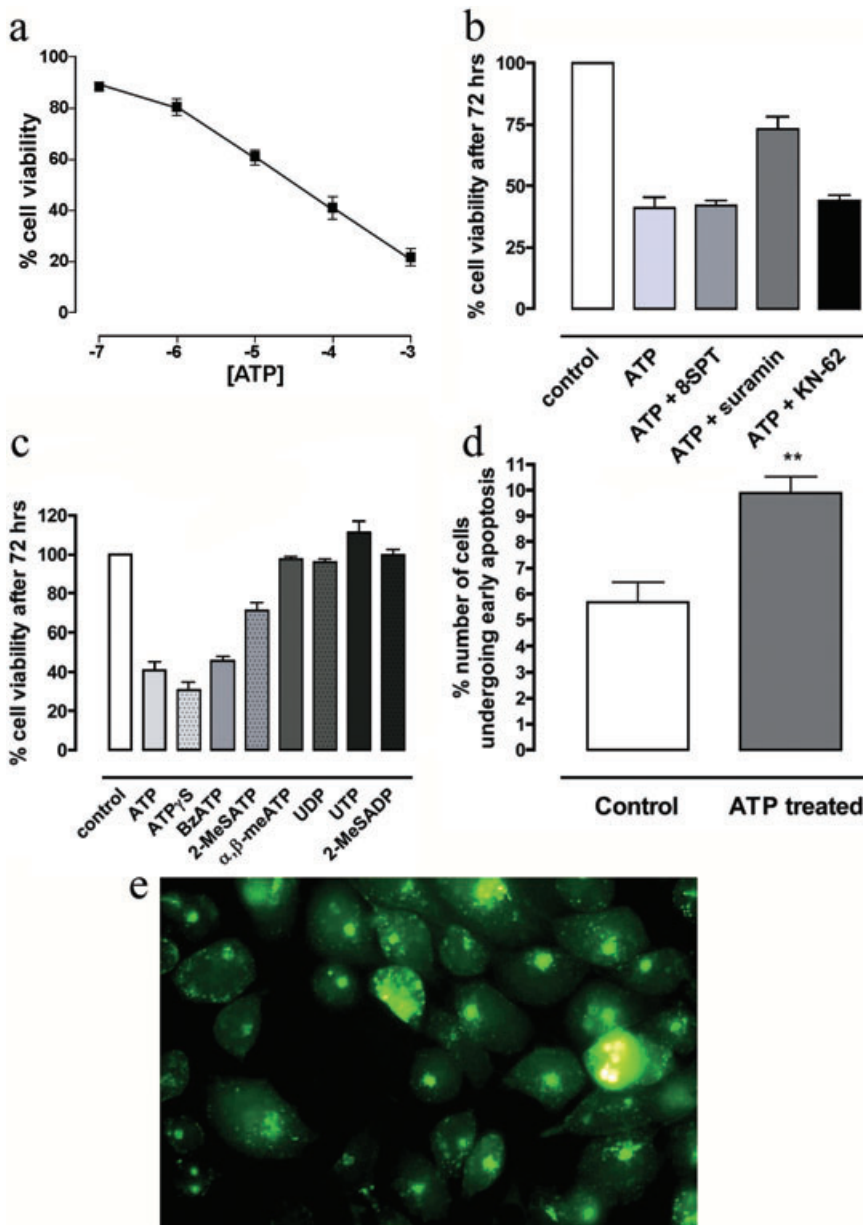
RESULTS

IN VITRO EXPERIMENTS

Using RT-PCR, the HT-1376 cells expressed mRNA for P2X_{4,5,7} and P2Y_{1,2,4,6,11} receptors (Fig. 1a,b).

The addition of exogenous ATP decreased cell viability in a dose-dependent manner (IC₅₀ 3.8×10^{-5} M \pm 5×10^{-6} M; Fig. 2a). One dose

FIG. 2. (a) Dose-response curve of the effect of ATP (10^{-7} – 10^{-3} M) on HT-1376 cell viability. (b) Effect of P2 receptor antagonists (10^{-4} M) on ATP-mediated growth inhibition. (c) Comparison of the effect on cell viability of various P2 receptor agonists on HT-1376 cells in vitro. (d) Bar chart showing the percentage number of cells undergoing early apoptosis (positive staining for annexin V) in control and ATP-treated (10^{-3} M) HT-1376 cells. ** $P < 0.001$. (e) Assessment of P2X₇ receptor pore formation using LY after incubation with ATP (10^{-3} M) at 37 °C for up to 1 h using fluorescence microscopy. No pore formation was identified.



of ATP (10^{-4} M) reduced cell viability by 59 (2.1)% after 72 h. The daily addition of ATP (10^{-4} M) for 72 h reduced cell viability by 88 (3)%. This was comparable to the growth inhibition seen with a single addition of ATP (10^{-3} M). This mimicked previous studies on ATP bioavailability in cell culture, which suggested that daily additions of lower concentrations of ATP maintain a minimum

threshold level for a short period (<24 h), which triggers growth inhibition similar to that seen with larger doses [7].

The addition of 8-SPT (10^{-6} – 10^{-4} M) had no effect on ATP-mediated growth inhibition, excluding any involvement of the ATP breakdown product, adenosine, via the activation of P1 receptors (Fig. 2b). The unlikely

involvement of adenosine was further confirmed by the greater efficacy seen with the hydrolysis resistant P2 agonist ATP γ S when compared with ATP (ATP γ S growth inhibition 102 (3.2)% vs 88.5 (4.4)% with ATP) (Fig. 2c).

Due to the relative paucity of selective purinergic receptor agonists and antagonists, pharmacological characterization of functional purinergic receptors is often by exclusion and assessment of relative orders of agonist potencies. The ability of suramin (10^{-6} – 10^{-4} M) to partially inhibit the antineoplastic action of ATP confirms the activation of P2 purinergic receptors, and makes activation of P2X₄ unlikely (Fig. 2b). The P2X_{1 and 3} receptor agonist α , β -meATP (10^{-7} – 10^{-4} M) had no effect on cell growth (Fig. 2c). The P2X₇ antagonist KN-62 (10^{-6} – 10^{-4} M) also had no effect on the ATP-mediated response (Fig. 2b). The P2Y receptor agonists 2-MeSADP (P2Y₁), UTP (P2Y₂, P2Y₄) and UDP (P2Y₆) (all 10^{-7} – 10^{-4} M) had no significant effect on growth inhibition, although UTP (10^{-7} – 10^{-4} M) had a significant mitogenic effect (percentage cell viability $111.3\% \pm 5.8\%$ after 72 h) (Fig. 2c). The final order of agonist potency was: ATP γ S > ATP > BzATP >> 2-MeSADP.

One addition of ATP (10^{-3} M) reduced the percentage cell viability (adherent cells) by 78.2 (2.2)% 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 undergoing early or late apoptosis. Staining of the remaining adherent cells showed a significant increase in the number of annexin V-positive cells compared with the control (apoptotic cells; 5.7 (0.79)% control vs 9.8 (0.62)% ATP-treated: *t*-test $P = 0.003$) indicating a significant increase in early apoptosis (Fig. 2d).

Activation of the pro-apoptotic P2X₇ receptor leads to the rapid formation of a large cell membrane pore with a molecular threshold of ≈ 900 Da. Its presence allows otherwise impermeable markers such as LY to traverse the cell membrane easily, which can be readily assessed using fluorescence microscopy. In the absence of P2X₇ receptor-gated pore formation, cellular LY uptake is by internalization into discrete intracellular vacuoles. By contrast, the presence of P2X₇ receptor-gated pores leads to the rapid and diffuse uptake of LY distributed evenly throughout the entire cell cytoplasm and

nuclear region. In our experiments, no P2X₇ receptor pore formation could be demonstrated after adequate incubation with ATP (10⁻³ M; Fig. 2e).

One addition of ATP (10⁻⁵ M) reduced cell viability by 32%. MMC also reduced cell viability in a dose-dependent manner (IC₅₀ 3.15 × 10⁻⁸ M ± 4.4 × 10⁻⁹ M). The combination of ATP (10⁻⁵ M) with MMC significantly shifted the MMC dose-response curve to the left (*P* = 0.007). Combined therapy reduced the dose at which half of the cells were killed by a factor of 10 (combined IC₅₀ 3.6 × 10⁻⁹ M ± 8.4 × 10⁻¹⁰ M). While the effect of drug combination was statistically significant (IC₅₀ MMC alone vs MMC + ATP; *P* = 0.003), assessment using Bliss synergy showed this effect to be additive only (Fig. 3).

IN VIVO EXPERIMENTS

When given each day immediately after tumour cell inoculation, ATP (25 mM) significantly reduced the growth of the implanted tumour by 64.3% after 35 days compared with the control (*P* = 0.003). No side-effects to treatment were noted. (Fig. 4a).

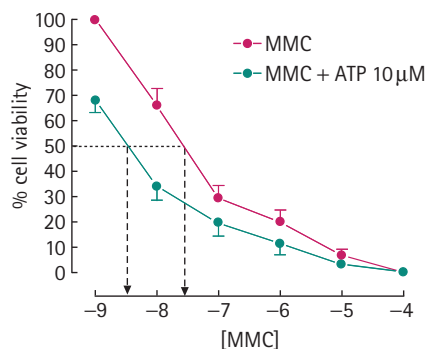
Daily i.p. injections of ATP (25 mM) significantly reduced the rate of established HT-1376 tumour growth compared with the control (*P* = 0.001). After 21 days treatment ATP reduced mean fractional tumour volume by 44.4% (Fig. 4b). There were no side-effects relating to treatment in any experimental group.

Histological analysis of the neoplasms in control mice using H&E staining and TEM showed tumours maintained the classical characteristics of urinary TCCs. While ATP-treated tumours were significantly smaller, light microscopy revealed no other histological changes. TEM detected an increase in both apoptotic bodies and necrosis in treated tumours (Fig. 5).

DISCUSSION

High-risk bladder cancer has an increased rate of tumour recurrence and disease progression and creates a dilemma in clinical management. Many urologists advocate a low threshold for radical cystectomy due to the unsatisfactory outcome of high-risk bladder

FIG. 3. Dose-response curve of the effect of combining ATP with MMC vs MMC alone on the viability of HT1376 cells *in vitro*.



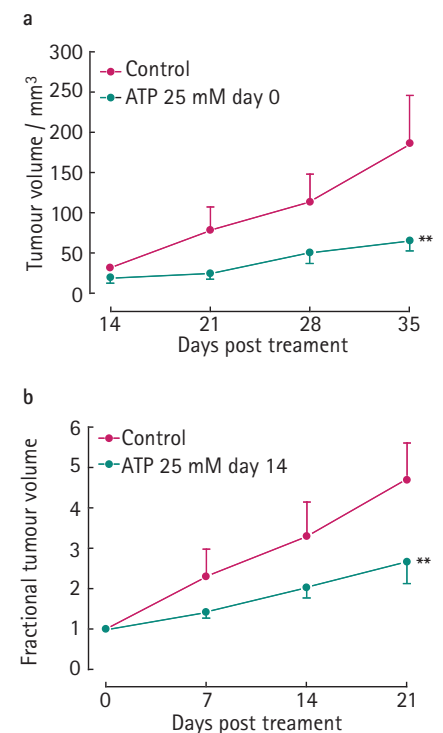
cancer with current treatment regimes. New therapies with an improved control of high-risk disease are therefore necessary to reduce the need for major surgery in such cases.

Extracellular nucleotides, such as ATP, have previously been shown to be involved in the control of cell growth in various malignancies including oesophageal [11], colon [12], ovarian [13] and hormone-refractory prostate cancers [7]. This trophic response has been found to be mediated via P2 receptor subtypes. Our aim was to assess the effect of ATP on high-grade bladder cancer, both *in vitro* and *in vivo*, and to determine which receptor subtypes were involved in any antineoplastic response that might be evident.

Using RT-PCR we identified the presence of several P2 receptor subtype mRNAs (P2X₄, P2X₅, P2X₇ and P2Y₁, P2Y₂, P2Y₆, P2Y₁₁). Using pharmacological characterization we were able to assess which of these receptors were functionally active in the inhibition of cancer cell growth.

The P1 receptor antagonist 8-SPT had no effect on the ATP-induced inhibition of cultured cell growth, showing that ATP was not acting on P1 receptors via its breakdown product, adenosine. However, the nonspecific P2 receptor antagonist, suramin, partially reversed ATP-mediated growth inhibition, confirming both a key role for P2 receptors and excluding the involvement of the P2X₄ receptor in the control of cell growth, as suramin is ineffective at this receptor [6]. The P2X₁ and P2X₃ receptor agonist, α,β-meATP, had no significant effect on cancer cell growth, confirming the lack of any functional role for these receptor subtypes.

FIG. 4. (a) Effect of daily i.p. ATP (25 mM) from day 0 on the growth of freshly implanted HT-1376 tumour cells *in vivo*. (b) Effect of daily i.p. ATP (25 mM) on the fractional growth of established HT-1376 tumour *in vivo* after 14 days initial growth.

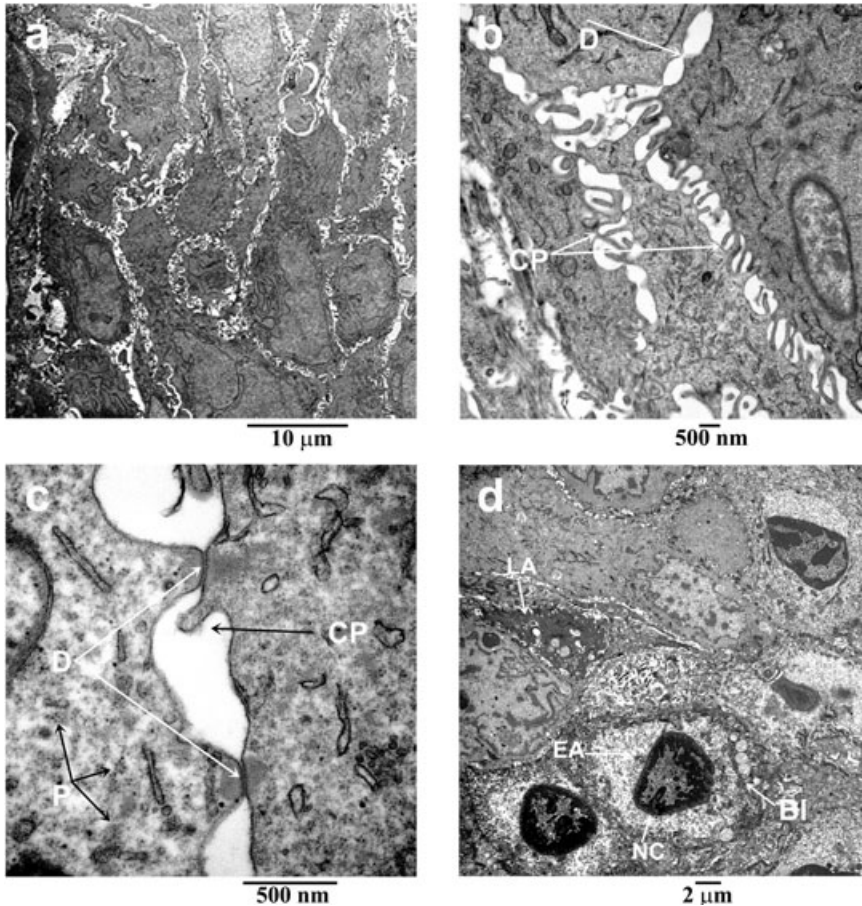


The P2X₇ receptor has previously been shown to be an important mediator of apoptosis in various cell types [6]. Confirmation of P2X₇ receptor mRNA in TCC cells raised hopes of a functional role for this receptor in the observed cell death. However, the selective P2X₇ receptor antagonist, KN-62, had no effect on the ATP-mediated growth inhibition, making the involvement of this receptor subtype seem unlikely, as did the assessment of fluorescent dye uptake (LY uptake) after incubation with ATP, as diffuse cellular uptake of LY did not occur.

The P2Y receptor agonists, 2-MeSADP (P2Y₁), UTP (P2Y₂, P2Y₄) and UDP (P2Y₆) exerted no antineoplastic effect, excluding any inhibitory role. UTP caused an overall increase in the number of viable cells, which is contrary to the findings of other groups investigating oesophageal and colon cancer [11,12], but concordant with the recognized growth-stimulatory effects of P2Y receptor subtypes as previously described [14].

The present data suggest that the antineoplastic action of ATP is mediated via

FIG. 5. Field view of ATP treated HT-1376 cells *in vivo*. The cells maintain the classical ultrastructural features of a human TCC with an increased nuclear : cytoplasm ratio and wide intracellular spaces, interrupted with short desmosomes (D) and interdigitating cytoplasmic processes (CP). Treatment results in cells undergoing early (EA) and late (LA) apoptosis with cell shrinkage, loss of cytoplasmic processes, nuclear chromatin condensation (NC), and cytoplasmic membrane blebbing (BI).



P2X₅ receptors, although the exact role of the P2Y₁₁ receptor cannot be defined due to the paucity of effective selective agonists and antagonists for this receptor subtype. This receptor profile is the same as that previously reported in the hormone-refractory prostatic cancer cell lines PC-3 and DU145 [7] although the bladder cancer cells were significantly more sensitive to the effects of ATP. These two malignancies differ in cellular type and origin (TCC vs prostate adenocarcinoma), and this finding might indicate a common target for the treatment of urological malignancies independent of cell type and origin. This is of particular interest given the increase rate of bladder cancer in patients with prostate cancer, and vice versa (18–19 fold increased rate) [15].

Growth inhibition occurs from either a decreased rate of proliferation, increased

apoptosis, or increased cell differentiation resulting in cells no longer able to continue the cell cycle. In the present study, ATP was able to significantly increase apoptosis after 72 h. Although studies have reported a potential differentiating role for P2X₅ [14] and P2Y₁₁ receptors [6], no studies have implicated these receptors in the induction of apoptosis. Apoptosis has classically been linked to the P2X₇ receptor, although we were unable to elicit a significant functional role for this receptor subtype. Ryten *et al.* [16] reported that activation of P2X₅ receptors mediated stimulation of cell differentiation markers and inhibited proliferation in skeletal muscle cells. It is therefore possible that the activation of P2X₅ receptors in bladder cancer might lead to cellular differentiation, resulting in cells unable to continue the cell cycle, which subsequently undergo apoptosis. This might explain the delay in apoptosis

detection, with no significant increase after 24 h incubation with ATP. ATP has previously been shown to induced S-phase arrest, which is cytostatic initially and with prolonged exposure resulted in cell death [17]. This might be due to ATP-treatment causing checkpoint defects in the arrested S-phase cells, preventing further cell cycle progression, and subsequent apoptosis in lethally damaged cells [18]. Assessment of cell differentiation using markers would help define the contribution of this process to the observed growth inhibition, and further clarify the antineoplastic mechanism of ATP in bladder cancer.

The primary principle of combined chemotherapy is to maximize antineoplastic activity while minimizing toxic side-effects of treatment. This is best achieved by combining drugs, which have different mechanisms of action and different patterns of resistance to minimize cross-resistance. ATP combined with MMC significantly, to increase its effect on cell death, reducing the chemotherapeutic drug concentration at which half of cells were killed, by a factor of 10. Assessment of the combined therapy using Bliss synergism showed that ATP combined with MMC in an additive manner only. The efficacy and toxicity of cell cycle nonspecific chemotherapeutic drugs such as MMC are related to their concentration. The use of ATP-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.

ATP treatment significantly reduced tumour growth *in vivo* without causing any adverse effects in the treated mice. The present results are similar to those described by Rapaport [8] who first used exogenous ATP in murine models of colon and pancreatic cancer and reported reductions in implanted tumour size. Previous studies have shown that *i.p.* injection of adenine nucleotides can lead to a sustained rise in blood and plasma ATP levels, lasting hours after the initial injection [8]. The sustained increases in blood ATP levels in athymic mice is due to cellular uptake and slow release of expanded ATP pools from normal, intact red blood cells [19]. The slow release of ATP from red blood cells gives rise to the sustained plasma concentration and subsequent increase in the extracellular concentration of ATP in this compartment.

Once the required threshold concentration for the antineoplastic activity of ATP is achieved cancer cell death can occur [7].

The concentration of ATP used in the present experiments to achieve a modest sustained increase in extracellular ATP pools in mice is relatively high. This is partly accounted for by the higher phosphomonoesterase activity in animal tissues compared with human tissue, which leads to the nonspecific breakdown of ATP. In addition, murine blood has significantly higher ecto-ATPase activity *in vitro* compared with human blood [19]. One would expect lower concentrations of ATP to achieve a similar sustained release and antineoplastic action in patients compared with the murine model. Any lowering of the required concentration would also reduce the potential side-effects from treatment, and increase tolerability in patients.

In patients with advanced lung cancer *i.v.* infusions of ATP have been used. In randomised trials, ATP treatment was well tolerated and effectively combated cancer cachexia [20]. ATP was taken up by red blood cells and released in a sustained manner in concurrence with murine studies. In patients with advanced stage IIIB non-small cell lung cancer, overall survival time increased from 9.3 months in the ATP-treated vs 3.5 months for the control, supporting the theory that ATP might treat the underlying malignancy as well as its systemic effects, although larger trials are needed to confirm this.

In conclusion, the present study shows, for the first time, that ATP has a significant antineoplastic action on high-grade bladder cancer acting via P2 receptors (P2X₅ and/or P2Y₁₁) both *in vitro* and *in vivo*. The advent of more specific P2X₅ and/or P2Y₁₁ receptor agonists would provide more specific therapeutic agents. Our results suggest that the two most common advanced urological malignancies might have a common therapeutic purinergic target despite their differing cellular origin.

CONFLICT OF INTEREST

None declared.

REFERENCES

- 1 Torti FM, Lum BL, Aston D *et al.* Superficial bladder cancer: the primacy of grade in the development of invasive disease. *J Clin Oncol* 1987; **5**: 125–30
- 2 Shelley MD, Wilt TJ, Court J, Coles B, Kynaston H, Mason MD. Intravesical bacillus Calmette–Guerin is superior to mitomycin C in reducing tumour recurrence in high-risk superficial bladder cancer: a meta-analysis of randomized trials. *BJU Int* 2004; **93**: 485–90
- 3 Filbeck T, Pichlmeier U, Knuechel R, Wieland WF, Rossler W. Reducing the risk of superficial bladder cancer recurrence with 5-aminolevulinic acid-induced fluorescence diagnosis. Results of a 5-year study. *Urologe A* 2003; **42**: 1366–73
- 4 Di Stasi SM, Giannantoni A, Stephen RL *et al.* Intravesical electromotive mitomycin C versus passive transport mitomycin C for high risk superficial bladder cancer: a prospective randomized study. *J Urol* 2003; **170**: 777–82
- 5 Burnstock G. Introduction: P2 receptors. *Curr Top Med Chem* 2004; **4**: 793–803
- 6 Burnstock G, Knight GE. Cellular distribution and functions of P2 receptor subtypes in different systems. *Int Rev Cytol* 2004; **240**: 31–304
- 7 Calvert RC, Shabbir M, Thompson CS, Mikhailidis DP, Morgan R, Burnstock G. Immunocytochemical and pharmacological characterisation of P2-purinoreceptor-mediated cell growth and death in PC-3 hormone refractory prostate cancer cells. *Anticancer Res* 2004; **24**: 2853–9
- 8 Rapaport E. Experimental cancer therapy in mice by adenine nucleotides. *Eur J Cancer Clin Oncol* 1988; **24**: 1491–7
- 9 Blaveri E, Simko JP, Korkola JE *et al.* Bladder cancer outcome and subtype classification by gene expression. *Clin Cancer Res* 2005; **11**: 4044–55
- 10 Webb JL. Effect of more than one inhibitor, antagonism, summation, and synergism. In Webb JL ed., *Enzyme and Metabolic Inhibitors*. New York: Academic Press, 1963: 488–512
- 11 Maaser K, Hopfner M, Kap H *et al.* Extracellular nucleotides inhibit growth of human oesophageal cancer cells via P2Y₂-receptors. *Br J Cancer* 2002; **86**: 636–44
- 12 Hopfner M, Maaser K, Barthel B *et al.* Growth inhibition and apoptosis induced by P2Y₂ receptors in human colorectal carcinoma cells: involvement of intracellular calcium and cyclic adenosine monophosphate. *Int J Colorectal Dis* 2001; **16**: 154–66
- 13 Schultze-Mosgau A, Katzur AC, Arora KK, Stojilkovic SS, Diedrich K, Ortman O. Characterization of calcium-mobilizing, purinergic P2Y₂ receptors in human ovarian cancer cells. *Mol Hum Reprod* 2000; **6**: 435–42
- 14 Greig AV, Linge C, Terenghi G, McGrouther DA, Burnstock G. Purinergic receptors are part of a functional signalling system for proliferation and differentiation of human epidermal keratinocytes. *J Invest Dermatol* 2003; **120**: 1007–15
- 15 Chun TY. Coincidence of bladder and prostate cancer. *J Urol* 1997; **157**: 65–7
- 16 Ryten M, Dunn PM, Neary JT, Burnstock G. ATP regulates the differentiation of mammalian skeletal muscle by activation of a P2X₅ receptor on satellite cells. *J Cell Biol* 2002; **158**: 345–55
- 17 Rapaport E. Treatment of human tumor cells with ADP or ATP yields arrest of growth in the S phase of the cell cycle. *J Cell Physiol* 1983; **114**: 279–83
- 18 Bunz F. Cell death and cancer therapy. *Curr Opin Pharmacol* 2001; **1**: 337–41
- 19 Rapaport E, Fontaine J. Anticancer activities of adenine nucleotides in mice are mediated through expansion of erythrocyte ATP pools. *Proc Natl Acad Sci USA* 1989; **86**: 1662–6
- 20 Agteresch HJ, Burgers SA, van der Gaast A, Wilson JH, Dagnelie PC. Randomized clinical trial of adenosine 5'-triphosphate on tumor growth and survival in advanced lung cancer patients. *Anticancer Drugs* 2003; **14**: 639–44

Correspondence: Geoffrey Burnstock, Autonomic Neuroscience Centre, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK. e-mail: g.burnstock@ucl.ac.uk

Abbreviations: MMC, mitomycin C; SBC, superficial bladder cancer; MEM, minimum essential medium; RT-PCR reverse transcriptase-PCR; bp, base pairs; 8-SPT, 8-thiophenyltheophylline; KN-62, 1-[N, O-bis (5-isoquinolinesulphonyl)-N-methyl-L-tryosyl]-4-phenylpiperazine; ATP_γS, adenosine 5'-O-(3 thiotriphosphate; BzATP, benzoyl-benzoyl ATP; 2-MeSATP, 2-methylthio ATP; 2-MeSADP, 2-methylthio ADP; α,β-meATP, α,β-methylene ATP; PI, propidium iodide; LY, lucifer yellow; H&E, haematoxylin and eosin; TEM, transmission electron microscopy; CF, cytotoxic fractions.