BJUI
BJU INTERNATIONAL

Effect of extracellular ATP on the growth of hormone-refractory prostate cancer *in vivo*

Majid Shabbir*[†], Cecil Thompson[‡], Michael Jarmulowiczc[§], Dimitri Mikhailidis[‡] and Geoffrey Burnstock[†]

*Departments of *Urology, [‡]Surgery and Clinical Biochemistry, [§]Histopathology, and [†]the Autonomic Neuroscience Centre, Royal Free and University College Medical School, London, UK*

Accepted for publication 29 November 2007

OBJECTIVE

To investigate whether the antineoplastic action of ATP on hormone-refractory prostate carcinoma (HRPC) cells *in vitro* also occurs *in vivo*, by examining the effect of ATP *in vivo* on tumours resulting from implanted HRPC cells in mice.

MATERIALS AND METHODS

HRPC tumour cells DU145 and PC-3 were implanted into male nude athymic mice. The effect of daily intraperitoneal (i.p.) injections of ATP (25 mM) on the growth of freshly implanted and established HRPC tumours was assessed. Histological examination using light and electron microscopy was used to confirm retention of the original ultrastructure of the implanted tumours.

RESULTS

Daily i.p. injections of ATP significantly reduced the growth of freshly implanted DU145 tumour by 57.8% ($P = 0.003$), and reduced the rate of growth of established DU145 tumour by 69.0% ($P = 0.006$). ATP also significantly reduced the growth of freshly implanted PC-3 tumour by 68.9% ($P < 0.001$). ATP treatment had no adverse effects on the host mice.

CONCLUSION

Our results show, for the first time, that ATP effectively reduces the growth of advanced HRPC tumours *in vivo*. This may represent a step in establishing ATP as an effective agent for HRPC treatment.

KEYWORDS

ATP, prostate, *in vivo*, hormone refractory disease

INTRODUCTION

Extracellular ATP has already been shown to effectively inhibit the growth of hormone-refractory prostate carcinoma (HRPC) cells *in vitro* via calcium-independent purinergic receptor-mediated apoptosis [1–3]. However, it is unclear whether this novel antineoplastic action in advanced prostate cancer extends *in vivo*. The establishment of *in vivo* efficacy is a vital prerequisite step to the potential future development of any new therapeutic agent.

There are numerous, well-established animal models of cancer, mostly involving the inoculation of human cancer cells into immunocompromised mice. T-cell deficient nude athymic mice have been reliably used in models of local tumour outgrowth after s.c. inoculation. In addition, this model can also be used to assess for metastatic spread, with the development of lymph node metastases in 60% and pulmonary metastases in 20% after s.c. inoculation of PC-3 cells [4]. While the use of severe combined immune deficiency (SCID) mice, and the orthotopic implantation of cancer cells has been shown to provide a better metastatic model [4,5], the severe immunodeficiency status makes these mice an extremely fragile and costly model to use in large numbers. In addition, orthotopic implantation models are limited by difficulties in assessing tumour progression without ending the experiment, as well as a higher risk of local complications, such as urinary retention in up to 40%, necessitating early termination of experiments [4]. Consequently, we chose to use a model of tumour outgrowth in nude athymic mice after s.c. inoculation.

Rapaport [6] first used extracellular ATP in nude athymic mice inoculated with human colon and pancreatic cancer cells in 1988. Exogenous ATP (50 mM) was given daily via i.p. injections into tumour-bearing mice. The mice were inoculated with either low or high tumour burdens. Exogenous ATP was effective at reducing the implanted tumour size, ranging from a complete cessation of tumour growth with low initial tumour burdens (0.5×10^3 cells), to a 31% reduction in models with higher initial tumour burdens (0.5×10^6) of the murine colon cancer cell line CT26. No adverse effect or toxicity was noted with the use of ATP in this report or in the studies that followed [7]. Subsequent to this, clinical trials in patients with cancer were carried out that have established systemic administration of ATP as a safe agent with the potential for being a useful anticancer agent [8–10].

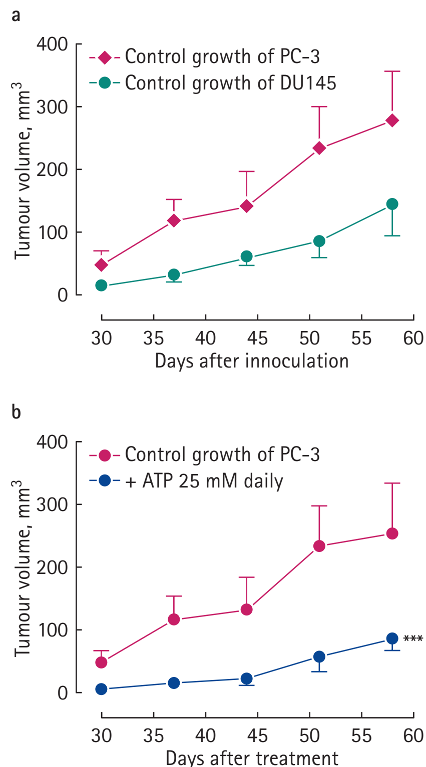
The aim of the present study was to examine the effect of ATP on HRPC *in vivo* on implanted DU145 and PC-3 tumour cells followed by i.p. injections of ATP.

MATERIALS AND METHODS

All cell lines were obtained from the European Collection of Cell Cultures (Salisbury, UK). PC-3 cells are HRPC cells established from bone metastases in a 62-year-old Caucasian male with high-grade (Gleason grade 4) prostatic adenocarcinoma [11]. DU145 cells are HRPC cells derived from a brain metastasis in a 69-year-old Caucasian male with Gleason grade 2 disease [12]. PC-3 cells were grown in Ham's F12K medium with 2 mM L-glutamine supplemented with 7.5% fetal calf serum (Sigma Chemical Co., Poole, UK) and a 1% antibiotic solution containing penicillin, streptomycin and amphotericin B (Sigma).

© 2008 THE AUTHORS

FIG. 1. (a) Comparison of implanted HRPC cell line tumour growth; (b) Effect of ATP (25 mM, i.p.) daily from day 0 on the growth of PC3 tumour cells in vivo.



DU145 cells were grown in minimum essential medium containing 2 mM L-glutamine supplemented with 10% fetal calf serum, 1% nonessential amino acid solution and a 1% antibiotic solution containing penicillin, streptomycin and amphotericin B (all Sigma). All cells were maintained in a humidified atmosphere at 37 °C containing 5% CO_2.

All experiments were conducted according to Home Office guidelines and under Home Office License with local Ethical Committee approval. Animal models were developed under license from the Home Office and ethically approved by local committee. Male 6–8-week-old nude athymic mice (MF1-nude; Harlen UK Ltd, Loughborough, UK) were used in all experiments. Mice were maintained in a specific pathogen-free environment, housed in individually ventilated cages (maximum five per cage), and given sterilized food and water *ad libitum*. Full gown, facemask, head cover and gloves were worn during any contact with the mice, and all procedures were performed aseptically in a pathogen-free environment. All mice were weighed at the start of the experiment and weekly thereafter. All mice were also assessed weekly for adverse events and well-being assessment scores were completed. Ten mice were used in each experiment, with five in each treatment and matched control arm of the study.

Tumours were induced by the s.c. inoculation of 1×10^6 cells of the relevant cell line (>90% viability) suspended in appropriate complete culture media and Matrigel (ratio 1:1, total inoculation volume 150 μL), as previously described by Sato *et al.* [13]. Matrigel, an extract of basement membrane proteins, induces rapid tumour development after s.c. injection and has been shown to improve reliable innoculation and growth of human tumours in athymic mice [14]. Pre-cooled syringes and needles were used in conjunction with Matrigel, which rapidly forms into a gel at room and body temperature. Cells were whirl mixed with Matrigel before injection into the right flank using a 20-G needle while the mice were lightly anaesthetized with halothane.

Mice were picked randomly and assigned to the two experimental treatment protocols used. In the first experiment, we assessed the effect of ATP treatment on freshly implanted tumours by administrating daily i.p. injections of ATP 25 mM (dissolved in 1 mL sterile saline, pH adjusted to 6.2 using NaOH [6]) starting the day after tumour inoculation. A control group received an equivalent volume of the vehicle (sterile saline) daily i.p. (five mice for each).

In the second experiment, the change in rate of growth of an established tumour after ATP treatment was assessed. The same therapeutic regime was used as before, but treatment was commenced once the tumour growth was well established (after 30 or 60 days, five mice for each) and continued for 28 days.

Tumour size was measured weekly using a standard calliper. Tumour volume was calculated in the first experiment using the standard formula:

$$\text{tumour volume} = \text{length} \times \text{width} \times \text{height} \times 0.52\ [15].$$

The rate of change of established tumour growth in the second experiment was calculated using the formula:

$$\text{fractional tumour volume} = (\text{volume on day measured}) / (\text{initial pretreatment tumour volume})\ [16].$$

At the end of the experiments, all mice were killed using increasing concentrations of CO_2 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 (fixed in 1.5% gluteraldeyde and post-fixed using osmium tetroxide, Embedded in Lemix Resin [Taab Laboratories Equipment Ltd, Aldermaston, UK] and cut into ultrathin sections before being viewed and photographed using a Philips CM120 transmission electron microscope). Tissue specimens were also taken from the lung, liver and axilla to assess for metastatic spread. Histological review of the pathology slides was performed in conjunction with a local histopathologist with a specialist interest in uro-oncology.

Data are expressed as mean (SEM). Cumulative response curves were plotted and compared using a two-way ANOVA followed by a *post hoc* (Bonferroni) test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

We initially established concurrent models using either PC-3 or DU145 cells injected s.c. to assess the pattern of cell growth and guide future treatment regimes. Both cell lines had an equal tumorigenicity with 100% tumour take. Tumour growth was more rapid in the PC-3 cell group, with a mean untreated tumour size 1.9-times greater than that seen in the DU145 model (Fig. 1a). Rapid growth with the PC-3 cell line increased the risk of skin breakdown over the implanted tumour with time. Therefore, DU145 cells were used in most of the tumour outgrowth models.

EFFECT OF ATP ON FRESHLY IMPLANTED PC-3 TUMOUR CELLS

One mouse from the control group failed to recover from the initial anaesthetic, reducing the number of mice in that group to four. All five mice in the treatment group survived the initial tumour implantation. Treatment with

© 2008 THE AUTHORS
JOURNAL COMPILATION © 2008 BJU INTERNATIONAL

FIG. 2. (a) Effect of ATP (25 mM, i.p.) daily from day 0 on the growth of DU145 tumour cells in vivo*; (b) Effect of ATP on the fractional growth of DU145 tumour cells* in vivo *after 30 days initial growth; (c) Effect of ATP on the fractional growth of DU145 tumour cells* in vivo *after 60 days initial growth; and (d) Effect of ATP on the growth of implanted DU145 tumour cells* in vivo *after 60 days initial growth; the lower mouse received ATP treatment vs no treatment in the upper mouse.*

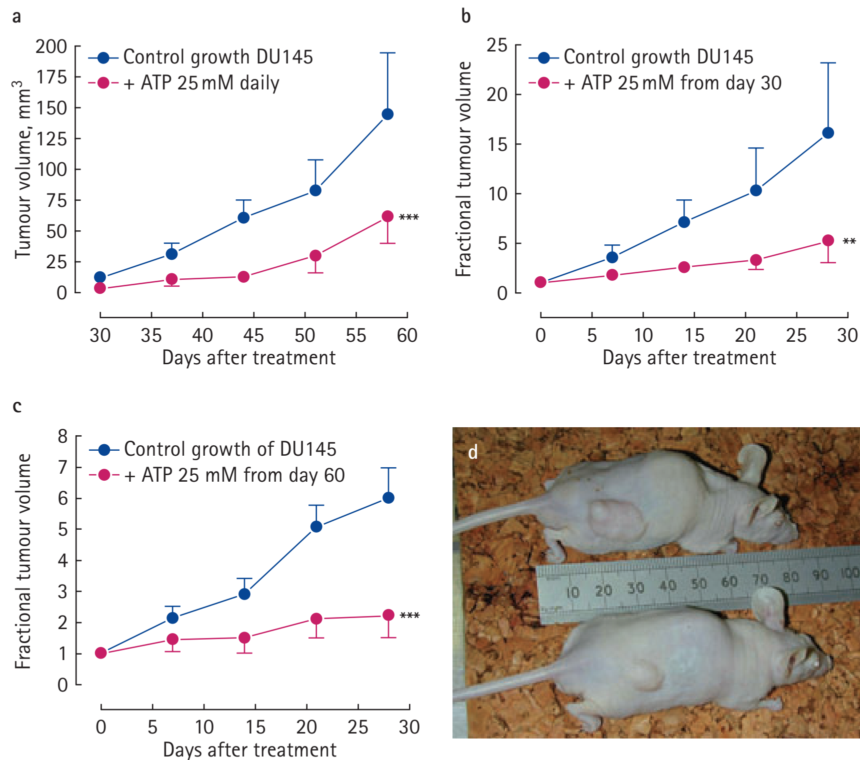
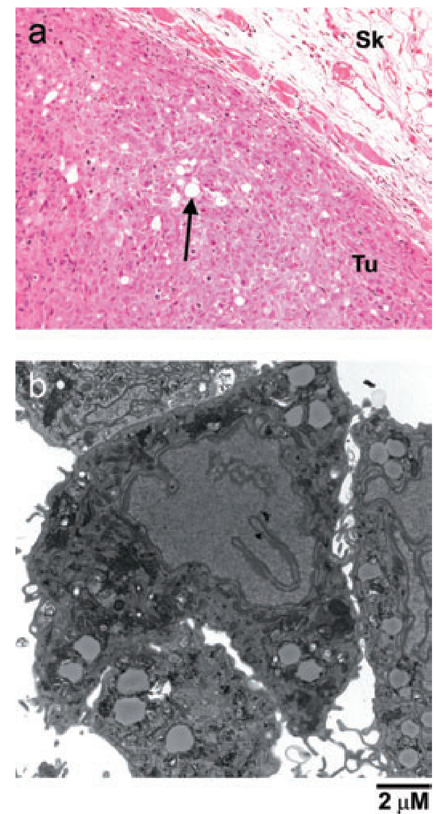


FIG. 3. (a) Microscopic examination of s.c. implanted DU145 prostate tumour (H&E, ×40). Sk, host skin; Tu, tumour. The tumour is histopathologically identical to human prostate cancer, Gleason grade 5 with tumour growth in solid sheets (as seen here) without forming any discernible glands. Note the punched-out circular lumens seen in this case (arrows). (b) Electron microscopic image of DU145 tumour cells grown in vivo. *Cells maintain the features of poorly differentiated tumour cells with a high nuclear : cytoplasmic ratio, dense cytoplasm with numerous ribosomes, and intracellular lipid droplets.*



ATP (25 mM) significantly reduced tumour growth throughout the experiment ($P < 0.001$; Fig. 1b). At the end of the experiment (day 58), ATP treatment had reduced the mean tumour volume by 68.9%.

EFFECT OF ATP ON FRESHLY IMPLANTED DU145 TUMOUR CELLS

One mouse from each cage (control and treatment) failed to recover from the initial anaesthetic, reducing the number of mice in each group to four. Treatment with ATP (25 mM) significantly reduced tumour growth throughout the experiment ($P = 0.003$; Fig. 2a). At the end of the experiment (day 58), ATP treatment had reduced the mean tumour volume by 57.8%, showing congruity with the results from the PC-3 model.

EFFECT OF ATP ON ESTABLISHED DU145 TUMOUR GROWTH

Daily i.p. injections of ATP (25 mM) significantly reduced the growth of established DU145 tumour after both 30 and 60 days initial growth. At the former time point, mean fractional tumour volume was reduced by 69.0% after ATP treatment (five mice) compared with the controls (four) after 28 days treatment ($P = 0.006$; Fig. 2b). At the latter time point, mean fractional tumour volume was reduced by 62.9% after ATP treatment (five mice) compared with the controls (four) after 28 days treatment ($P < 0.001$; Fig. 2c,d).

Histological examination of the induced tumour using H&E staining showed growth of tumour that was histopathologically identical to human prostate cancer, Gleason grade 5, with tumour growth in solid sheets without forming any discernible glands (Fig. 3a). This pattern of tumour growth was an up-regulation from the initial DU145 tumour, which was Gleason grade 2 when first isolated. Further histological examination of the tumour outgrowth using transmission electron microscopy confirmed that the implanted cells retained the microscopic features of a poorly differentiated adenocarcinoma (Fig. 3b). These findings increase the validity of the results from the *in vivo* model, and confirmed the implanted tumour retained the features of the original human tumour.

DISCUSSION

The use of cancer murine models is important in the development of new therapeutic methods for advanced malignancies. In the present study we used tried and tested models of tumour outgrowth after s.c. cell inoculations. Using the DU145 and PC-3

© 2008 THE AUTHORS
JOURNAL COMPILATION © 2008 BJU INTERNATIONAL

HRPC cells, we were able to show that i.p. injections of ATP significantly and effectively reduced the growth of both freshly implanted and established tumour outgrowths by 57–69%. No mice showed side-effects or complications at any stage of the study related to ATP treatment, and body weight was maintained throughout. Using a combination of light and electron microscopy, we were able to confirm that the inoculated tumour cells retained their original morphology and cellular characteristics, further validating the relevance of these results to the expected effects of ATP treatment for tumours in patients.

The present results extend those of Rapaport [6] who first used exogenous ATP in murine models of colon and pancreatic cancer to achieve reductions in implanted tumour size of 31–43% after 15–22 days treatment with ATP (50 mM). Although the treatment time was shorter, the maximum tumour burden used was 50% less than those used in the present experimental protocols. Despite the greater tumour burdens in the present models, the lower dose of ATP (25 mM) reduced tumour growth more effectively (57–69%).

Previous studies have shown that the i.p. injection of extracellular ATP can lead to a sustained rise in blood and plasma ATP levels lasting for hours after the initial injection. Rapaport [6] found that i.p. injections of ATP (50 mM) led to a 3–4-fold increase in baseline plasma ATP levels at 4 h, with sustained increases lasting 5–18 h after injection. This sustained increase in murine blood ATP levels has been shown to be due to cellular uptake and slow release of expanded ATP pools from normal, intact red blood cells [17]. This same phenomenon has been shown in patients treated with i.v. ATP, with a 50–70% increase in ATP concentrations above baseline maintained at 24 h with a similar sustained release mediated by red blood cells [18]. As *in vitro* experiments have previously shown that the antineoplastic action of ATP is perpetuated once a required threshold concentration is achieved and maintained, the uptake and sustained release of ATP from red blood cells may play a key mechanistic role [19].

The concentration of ATP used in the present murine model to achieve modest, sustained increases in extracellular ATP pools is relatively high compared with concentrations usable in patients. 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 [20]. In addition, murine blood has been shown to have significantly higher ecto-ATPase activity *in vitro* compared with human blood [17,21]. The lower concentrations of ATP necessary to achieve a similar sustained release and antineoplastic action in patients would also reduce the potential side-effects from treatment, and increase tolerability in patients.

Patients with lung cancer have safely been given i.v. ATP. Agteresch *et al.* [22] investigated the pharmacokinetics of i.v. ATP in 28 patients. Treatment was well tolerated with no side-effects in 64% of the group. In those who did develop side-effects, the most common was chest tightness (15%) or dyspnoea (10%), which was mild (level 1 or 2 by USA 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 that study concentrations of 25–75 mg/kg were used.

In keeping with murine models, ATP treatment has been shown to maintain body weight and decrease cancer cachexia in human studies [22]. In the murine cancer models, i.p. ATP inhibited weight loss in the mice with advanced tumour growth independent of its primary antineoplastic action. This anticachectic effect was thought to occur primarily via the ATP breakdown product, adenosine, which had little antineoplastic activity, but was effective at reducing weight loss. However, the anticachectic effect of ATP was greater than that seen with adenosine alone, implying that some other mechanism must be involved, at least in part [17]. In their trial, Agteresch *et al.* [22] found i.v. 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.

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 tumour and the systemic side-effects of the tumour in a patient with advance disease, as shown in the murine *in vivo* models. This could potentially have a considerable impact on the management of patients with advanced HRPC.

ACKNOWLEDGEMENTS

The authors thank Dr Gillian E. Knight for editorial assistance.

CONFLICT OF INTEREST

None declared.

REFERENCES

1 **Shabbir M, Ryten M, Thompson C, Mikhailidis DP, Burnstock G.** Characterisation of calcium-independent purinergic receptor-mediated apoptosis in hormone refractory prostate cancer. *BJU Int* 2008; **101**: 352–9
2 **Fang W-G, Pirnia F, Bang YJ, Myers CE, Trepel JB.** P_2-purinergic receptor agonists inhibit the growth of androgen-independent prostate carcinoma cells. *J Clin Invest* 1992; **89**: 191–6
3 **Janssens R, Boeynaems JM.** Effect of extracellular nucleotides and nucleoside on prostate carcinoma cells. *Br J Pharmacol* 2001; **132**: 536–46
4 **Rembrink K, Romijn JC, van der Kwast TH, Rubben H, Schroder FH.** Orthotopic implantation of human prostate cancer cell lines: a clinically relevant animal model for metastatic prostate cancer. *Prostate* 1997; **31**: 168–74
5 **Shibayama T, Tachibana M, Deguchi N, Jitsukawa S, Tazaki H.** SCID mice: a suitable model for experimental studies of urologic malignancies. *J Urol* 1991; **146**: 1136–7
6 **Rapaport E.** Experimental cancer therapy in mice by adenine nucleotides. *Eur J Cancer Clin Oncol* 1988; **24**: 1491–7
7 **White N, Burnstock G.** P2 receptors and cancer. *Trends Pharmacol Sci* 2006; **27**: 211–7
8 **Haskell CM, Wong M, Williams A, Lee LY.** Phase I trial of extracellular adenosine 5′-triphosphate in patients with advanced cancer. *Med Pediatr Oncol* 1996; **27**: 165–73
9 **Haskell CM, Mendoza E, Pisters KM, Fossella FV, Figlin RA.** Phase II study of intravenous adenosine 5′-triphosphate in patients with previously untreated stage

© 2008 THE AUTHORS
JOURNAL COMPILATION © 2008 BJU INTERNATIONAL

IIIB and stage IV non-small cell lung cancer. *Invest New Drugs* 1998; **16**: 81–5

10 **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

11 **Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW.** Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* 1979; **17**: 16–23

12 **Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF.** Isolation of a human prostate carcinoma cell line (DU 145). *Int J Cancer* 1978; **21**: 274–81

13 **Sato N, Gleave ME, Bruchovsky N, Rennie PS, Beraldi E, Sullivan LD.** A metastatic and androgen-sensitive human prostate cancer model using intraprostatic inoculation of LNCaP cells in SCID mice. *Cancer Res* 1997; **57**: 1584–9

14 **Fridman R, Kibbey MC, Royce LS *et al.*** Enhanced tumor growth of both primary and established human and murine tumor cells in athymic mice after coinjection with Matrigel. *J Natl Cancer Inst* 1991; **83**: 769–74

15 **Gleave M, Hsieh JT, Gao CA, von Eschenbach AC, Chung LW.** Acceleration of human prostate cancer growth *in vivo* by factors produced by prostate and bone fibroblasts. *Cancer Res* 1991; **51**: 3753–61

16 **Ahmed S, Johnson CS, Rueger RM, Trump DL.** Calcitriol (1,25-dihydroxycholecalciferol) potentiates activity of mitoxantrone/dexamethasone in an androgen independent prostate cancer model. *J Urol* 2002; **168**: 756–61

17 **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

18 **Agteresch HJ, Dagnelie PC, Rietveld T, van den Berg JW, Danser AH, Wilson JH.** Pharmacokinetics of intravenous ATP in cancer patients. *Eur J Clin Pharmacol* 2000; **56**: 49–55

19 **Calvert RC, Shabbir M, Thompson CS, Mikhailidis DP, Morgan RJ, Burnstock G.** Immunocytochemical and pharmacological characterization of P2-purinoreceptor-mediated cell growth and death in PC-3 hormone refractory prostate cancer cells. *Anticancer Res* 2004; **24**: 2853–60

20 **LePage GA, Lin YT, Orth RE, Gottlieb JA.** 5′-Nucleotides as potential formulations for administering nucleoside analogs in man. *Cancer Res* 1972; **32**: 2441–4

21 **Trams EG.** A proposal for the role of ecto-enzymes and adenylates in traumatic shock. *J Theor Biol* 1980; **87**: 609–21

22 **Agteresch HJ, Dagnelie PC, van der Gaast A, Stijnen T, Wilson JH.** Randomized clinical trial of adenosine 5′-triphosphate in patients with advanced non-small-cell lung cancer. *J Natl Cancer Inst* 2000; **92**: 321–8

Correspondence: Professor 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: **HRPC**, hormone-refractory prostate carcinoma; **SCID**, severe combined immune deficiency; **H&E**, haematoxylin and eosin.

© 2008 THE AUTHORS
JOURNAL COMPILATION © 2008 BJU INTERNATIONAL