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

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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 ml/kg) 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 ml/kg) 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 × 106 cells), to a 31% reduction in models with higher initial tumour burdens (0.5 × 107 cells) 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 ml/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).
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 inoculation 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 caliper. Tumour volume was calculated in the first experiment using the standard formula:

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

The rate of change of established tumour growth in the second experiment 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 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% gluteraldehyde 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
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 HE 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...
ATP (50 mm) 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 anticaesthetic 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 anticaesthetic 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.

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CONFLICT OF INTEREST

None declared.

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