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REVIEW ARTICLE

Purinergic signalling and cancer

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Abstract Receptors for extracellular nucleotides are widely expressed by mammalian cells. They mediate a large array of responses ranging from growth stimulation to apoptosis, from chemotaxis to cell differentiation and from nociception to cytokine release, as well as neurotransmission. Pharma industry is involved in the development and clinical testing of drugs selectively targeting the different P1 nucleoside and P2 nucleotide receptor subtypes. As described in detail in the present review, P2 receptors are expressed by all tumours, in some cases to a very high level. Activation or inhibition of selected P2 receptor subtypes brings about cancer cell death or growth inhibition. The field has been largely neglected by current research in oncology, yet the evidence presented in this review, most of which is based on in vitro studies, although with a limited amount from in vivo experiments and human studies, warrants further efforts to explore the therapeutic potential of purinoceptor targeting in cancer.

Keywords P2 receptors · Extracellular ATP · Cell growth · Apoptosis · Cancer · Anti-cancer drugs

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Introduction

Purinergic signalling, where adenosine 5′-triphosphate (ATP) and adenosine act as extracellular signalling molecules, was first proposed in 1972 [1]. Later, receptors for purines and pyrimidines were cloned and functionally characterised (see [2]). Four subtypes of P1 (adenosine) receptors (A_1, A_{2A}, A_{2B} and A_3), seven subtypes of P2X ion channel receptors (P2X1-7) and eight subtypes of G protein-coupled receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) have been identified (see [3]). These receptors are expressed by most non-neuronal cell types as well as neurons and their physiological roles have been explored (see [4]). In recent years, there have been a number of studies of the pathophysiological roles of purinergic signalling and its therapeutic potential for a variety of diseases (see [5, 6]).

There is growing interest in the therapeutic potential of purinergic signalling for the treatment of cancer (see reviews by [7–17]). The anti-neoplastic activity of ATP was first shown by Rapaport in 1983 [18] (see also [19–21]), who demonstrated that the addition of exogenous ATP to adenocarcinotomous pancreatic and colon cancer cells inhibited cell growth by causing cell cycle arrest in the S phase. In contrast, adenosine has been suggested to promote tumour growth (see [22]). Adenocarcinomas are malignant epithelial tumours arising from glandular structures which are constituent parts of most organs of the body. Subsequent studies have shown an anti-neoplastic action of extracellular nucleotides in colorectal cancer [23], leukaemia [24, 25], oesophageal cancer [26], Ehrlich ascites tumour cells [27], squamous cell skin cancer [28], lung cancer [29], cervical cancer [30], H35 hepatoma cells [31], prostate cancer [32], bladder cancer [33], retinoblastoma [34], neuroblastoma [35], glioma [36] and melanoma [37, 38]. Tumour cells have very high ATP content compared to most healthy cells [39, 40]. ATP-depleting strategies enhance anti-cancer agent activity [41]. Tumour progression was inhibited in ecto-5′-nucleotidase (CD73)-deficient mice [42], while

vascular ectonucleoside triphosphate diphosphohydrolase (CD39) directly promoted tumour cell growth [43]. It has been suggested that NTPDase6 may be a tumour suppression gene and a determinant of cisplatin resistance in testicular cancer [44].

While it is generally acknowledged that treatment with ATP or ATP analogues has a strong cytotoxic effect on several tumours, it is also clear that low ATP doses (as occurs, for example, during spontaneous release of this nucleotide from virtually every cell type) have a growth-promoting effect. Depending on the P2 receptor subtypes expressed, tumour cells may be more sensitive to the death inducing or to the trophic effect of ATP. This observation underscores the need for an in-depth characterization of P2 receptors in tumour cells, in order to fully recognise the potential of purinergic signalling in cancer therapy.

Different P2 purinergic receptor subtypes are involved in the growth inhibitory response observed in the different malignant cell types challenged with ATP or other nucleotides. 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. It is likely that the final effect is due to a combination of multiple effects due to stimulation of more than one P2 receptor subtype. To date, five P2 receptor subtypes have primarily been implicated in the growth inhibition of cancer cells, namely P2X5, P2X7, $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ [10], 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 [45] and squamous cell skin cancer [28]. In human oesophageal and colorectal cancer cells, $P2Y_2$ receptor stimulation results in apoptotic cell death [23, 26], while in melanoma, stimulation of the same receptor increases cell proliferation [45]. The explanation for these divergent responses remains unclear at present. Embryonic carcinoma cells are widely used models for studying the mechanisms of proliferation and differentiation occurring during early embryogenesis. A recent investigation has shown that down-regulation of P2X2 and P2X7 receptor expression by RNA interference affects phenotype specification of P19 embryonal carcinoma cells [46].

In the HL-60 human leukaemic cell line, P2X receptor-mediated events result in growth inhibition [25]. P2X7 receptors induce apoptosis in melanoma [45], squamous cell skin cancer [28], lung cancer [29] and cervical cancer [30] (and see [47]). The P2X7 receptor is most widely accepted as the purinergic receptor mediator of apoptotic or necrotic cell death, as initially suggested by early experiments in mouse tumour cell lines where ATP was shown to trigger cell death via a necrosis or apoptosis, depending on the cell type [48, 49]. Whether this is due to preferential expression by different mouse tumour cells of different truncated P2X7 splice variants is not currently known. Analysis of the effect of the P2X7 receptor on tumour growth is made more complex by the observation that tonic, as opposed to pharmacological, stimulation may have a trophic, growth-promoting, rather than cytotoxic effect [50]. This intriguing effect of P2X7 receptors has been recently shown to be present also in mouse embryonic stem cells [51] and the intracellular signalling pathways have been identified [14, 52]. Besides cell growth, there is

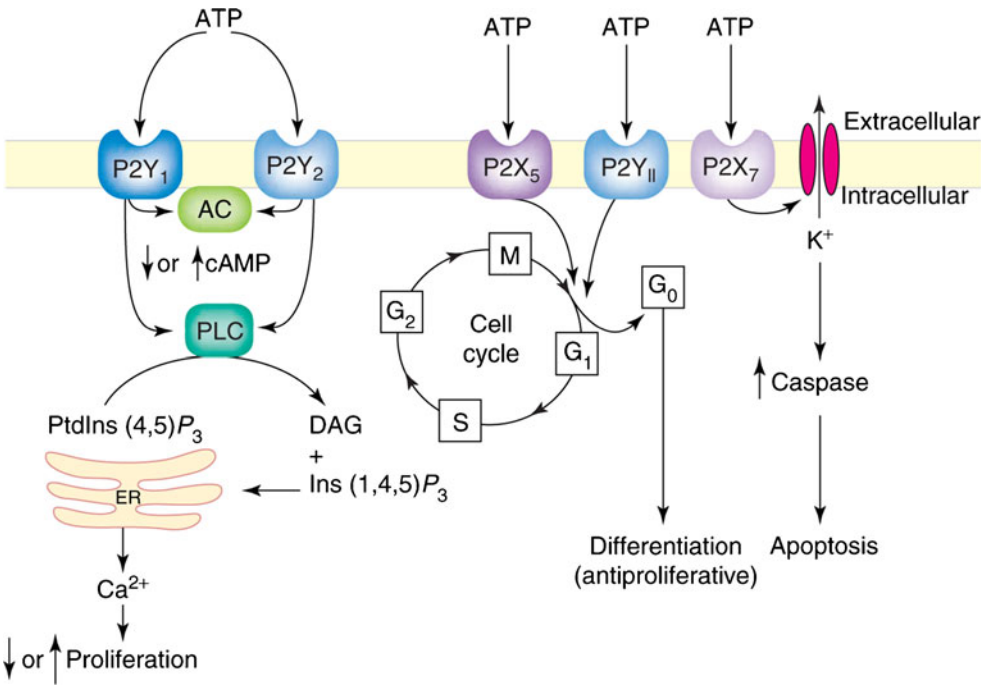

Fig. 1 Schematic diagram illustrating the different mechanisms by which P2 receptor subtypes might alter cancer cell function. $P2Y_1$ and $P2Y_2$ receptors could affect the rate of cell proliferation through altering the intracellular levels of cAMP by modulating adenylyl cyclase (*AC*) or by increasing intracellular calcium levels through the phospholipase C (*PLC*) pathway. P2X5 and $P2Y_{11}$ receptor activation might switch the cell cycle from proliferation into a state of differentiation. The P2X7 receptor activates the apoptotic caspase enzyme system. (Reproduced from [10] with permission.)

Table 1 Examples of P2 receptor subtype expression in different cancers

Cancer type	Primary tissue	Cell line	mRNA	Protein	Second messenger system/ functional response	Change in cell number	References
Melanoma	Yes	A375	P2X7 $P2Y_1$ $P2Y_2$ $P2Y_4$ $P2Y_6$	P2X7 $P2Y_1$ $P2Y_2$ $P2Y_4$ $P2Y_6$	Caspase 3/7 PLC-mediated $[Ca^{2+}]_i$	↑ $P2Y_2$ ↓ $P2Y_1$ P2X7	[37, 45]
Skin (squamous cell carcinoma)	Yes	A431	Not investigated	P2X5 P2X7 $P2Y_1$ $P2Y_2$	Caspase 3/TUNEL PCNA	↑ $P2Y_2$ ↓ $P2Y_1$ P2X5 P2X7	[28]
Colorectal	Yes	HT29	P2X1 P2X4 P2X5 P2X7	P2X1 P2X4 P2X7	$[Ca^{2+}]_i$	↑ or ↓ $P2Y_2$	[209]
		HCT8 CaCo-2	$P2Y_1$ $P2Y_2$ $P2Y_4$ $P2Y_6$	$P2Y_1$ $P2Y_2$	Cell Death ELISA	↓ $P2Y_1$ P2X7	[771]
Oesophageal	Yes	Kyse-140	P2X4 P2X5 $P2Y_2$	$P2Y_2$	Caspase 3 PLC-mediated $[Ca^{2+}]_i$ TUNEL	↓ $P2Y_2$	[26]
Lung	No	A459	$P2Y_2$ $P2Y_6$	Not investigated	CaMKII NF-κB $[Ca^{2+}]_i$	↑ $P2Y_2$ $P2Y_6$	[29]
Prostate	No	PC-3 DU145	P2X4 P2X5 P2X7 $P2Y_1$ $P2Y_2$ $P2Y_6$ $P2Y_{11}$	P2X4 P2X5 P2X7 $P2Y_2$	PLC-mediated $[Ca^{2+}]_i$ TUNEL	? $P2Y_2$ ↓ P2X5 P2X7	[58, 106, 173]
Brain tumours	No	1321N1 C6 U-251MG	$P2Y_1$ $P2Y_{12}$	$P2Y_1$	Caspase 3 ERK PLC-mediated $[Ca^{2+}]_i$	↑ or ↓ $P2Y_1$ ↑ $P2Y_{12}$	[589, 610, 637]
		U138-MG U-87MG			Adenylyl cyclase G_i		
Cervical	Yes	CaSki	Not investigated	P2X4 P2X7 $P2Y_2$	Caspase 9/TUNEL	↓ P2X7	[30]
Breast	No	MCF-7 Hs578T SK-Br3	$P2Y_2$ P2X7	P2X7	$[Ca^{2+}]_i$ $[K^+]_i$	↑ $P2Y_2$	[106, 114, 141, 186]
		T47-D					
		MDA-MB-231					
Ovarian	No	OVCAR-3 EFO-21	$P2Y_2$	Not investigated	$[Ca^{2+}]_i$	↑ or ↓ $P2Y_2$	[186, 417]
		EFO-27					
Endometrial	No	HEC-1A Ishikawa	$P2Y_2$	Not investigated	PLC-mediated $[Ca^{2+}]_i$	↑ $P2Y_2$	[431]
Haematological malignancies	Yes	HL-60 NB-4	P2X7 $P2Y_{11}$	P2X7	$[Ca^{2+}]_i$ Adenylyl cyclase PKA	↓ P2X7	[452, 463, 772]
Bladder (TCC)	No	HT-1376	Not investigated	Not investigated	Not investigated	↓ $P2Y_{11}$ P2X4 P2X5	[33]
Thyroid	No	ARO	$P2Y_1$ $P2Y_2$	Not investigated	PLC-mediated $[Ca^{2+}]_i$	↑ $P2Y_1$ $P2Y_2$	[358]

Upwards arrow indicates that stimulation of receptor subtype results in an increase in cell numbers and downwards arrow indicates that stimulation of receptor subtype results in a decrease in cell numbers. Question mark indicates inconclusive or contradictory data. (Reproduced from [10] with permission.)

CaMKII calmodulin-dependent protein kinase II, *ERK* extracellular signal-regulated kinase, *NF-kB* nuclear factor kB, *PCNA* proliferating cell nuclear antigen, *PKA* protein kinase A, *PLC* phospholipase C, *TUNEL* terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labelling

evidence from in vitro and in vivo studies that P2X7 might also participate in metastatic dissemination [53, 54]. In epithelia originating from the ectoderm, urogenital sinus and the distal paramesonephric duct, decreased expression of P2X7 receptors precedes or coincides with neoplastic development [55]. An endogenously expressed truncated P2X7 receptor lacking the C-terminus was shown to be preferentially upregulated in epithelial cancer cells, but fails to mediate pore formation and apoptosis [56]. The cell differentiating effects of $P2Y_{11}$ receptors in leukaemia cells [57] and P2X5 receptors in skeletal muscle cells [18] and keratinocytes [58] may induce alterations to normal cell cycle progression and promote cell death.

Microarray analysis of lung, breast, prostate and gastric cancers as well as melanoma revealed a significantly higher expression of A_{2B} and P2Y receptors [59]. A_3 receptors have also been shown to be highly expressed in tumour compared to normal cells [60]. Surprisingly, proliferation of most tumour cells is inhibited by adenosine, although it promotes cell proliferation via A_2 receptors in human epidermoid carcinoma cells. NMR structure and functional characterisation of a human nucleoside triphosphatase involved in human tumorigenesis have been described [61]. Neuroendocrine tumours predominantly express A_{2A} and A_{2B} receptors and their activation leads to increased proliferation and secretion of chromogranin A [62]. One of the crucial issues to understand host–tumour interactions is the biochemical composition of the tumour microenvironment. In vivo studies show that the extracellular milieu of solid tumours has high adenosine content [63]. Due to the well-known immunosuppressive activity of adenosine, this finding gives a crucial hint for the understanding of immunoescape strategies of cancer. The possibility was raised that adenosine may act as an inhibitor of killer T cell activation in the microenvironment of solid tumours [64]. More recently, chimeric plasma membrane-targeted luciferase revealed high extracellular ATP concentrations (in the hundreds micromolar range) in tumours but not tumour-free tissues [65]. Therefore, it seems that the tumour microenvironment is a site of active extracellular ATP release/generation and conversion to adenosine, thus producing a milieu rich in growth-promoting and immunomodulatory factors. Not surprisingly, the inflammatory microenvironment is also very rich in extracellular ATP [66].

It was suggested early that adenosine may regulate the vascular supply to neoplastic tissue and thereby influence the growth of tumours [67]. The major blood vessels that supply tumours are innervated by sympathetic nerves (that release ATP as a cotransmitter with noradrenaline (NA)), but the newly formed blood vessels within tumours are not innervated [68–71]. It has been suggested that P2 purinoceptor antagonists may inhibit neovascularisation in tumour growth and metastases [72]. Inhibition of tumour angiogenesis by targeting endothelial surface ATP synthase with sangivamycin, an anti-tumour agent, was reported [73]. It has been speculated that cancer cells affect endothelial cells during metastasis, perhaps involving P2Y receptor-mediated increases in $[Ca^{2+}]_i$ [74].

There is compelling evidence that tumour cells of various kinds release substantial amounts of ATP in response to mechanical deformation, hypoxia and some agents, as well as following necrosis and ischaemia [75, 76]. There is a correlation between levels of ATP in tumour cells and the development of cancer: ATP-depleting agents can markedly enhance cancer therapy (see [77, 78]). Cancer therapy by endogenous or transferred anti-tumour T cells has been used complementary to conventional cancer treatment by surgery, radiotherapy or chemotherapy. However, this approach is limited because tumours can create a hostile immunosuppressive microenvironment that prevents their destruction by anti-tumour T cells (see [79]). However, genetic deletion of immunosuppressive A_{2A} receptors or the use of A_{2A} antagonists can prevent the inhibition of anti-tumour T cells by the tumours, thus opening up a novel therapeutic approach to cancer immunotherapy [63, 80]. Chemotherapy induces ATP release from tumour cells, which leads to apoptotic cell death [81], probably via P2X7 receptors. Studies have shown that certain chemotherapeutic drugs, such as anthracyclines, are potent inducers of immunogenic cancer cell death, thereby triggering anti-tumour immune responses [82]. It was hypothesised that the inflammasome may contribute a third level regulation of immunogenic chemotherapy and that the release of ATP from dying tumour cells is involved in the activation of the inflammasome. Chemotherapeutic drugs such as cadmium, etoposide, mitomycin C, oxaliplatin, cisplatin, staurosporine, thapsigargin, mitoxanthrane and doxorubin may trigger release of ATP from tumour cells before and during apoptosis [81] and also from dendritic cells (DCs) [83]. Mice deficient in P2X7 receptor-expressing tumours failed to respond to oxaliplatin treatment and failed to mount tumour-specific CD8-T cell responses. In this process, the increase of ATP concentration within the tumour microenvironment is crucial, as ATP stimulates the P2X7 receptor of DCs to drive secretion of the key pro-inflammatory cytokine interleukin (IL)-1β. This cytokine potentiates antigen presentation to $CD4^+$ lymphocytes, thus enhancing the anti-tumour immune response. As emphasised in this 'Introduction', the ATP level is a crucial determinant for the final outcome, since while high ATP doses will potentiate anti-tumour immunity, low ATP levels are likely to be immunosuppressive, as shown by the finding that human DCs stimulated by low ATP concentrations produce less pro-inflammatory cytokines, more IL-10 and synergize with interferon to upregulate indoleamine 2,3-dioxygenase levels [84]. More recently, in vivo experiments have shown that release of ATP from cancer cells is associated with autophagy, a protective mechanism in cancer, and that the increase in the pericellular environment is essential for a proper

anti-cancer immunoresponse and for the efficacy of chemotherapy [85].

The possibility has been raised that ATP may be used for the treatment of both a primary tumour and the systemic side effects of the tumour in patients with advanced disease, as demonstrated in murine in vivo models. This could potentially have a considerable impact on the management of patients with advanced malignancy.

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 trialled in patients with lung cancer. A phase I trial for extracellular ATP in patients with advanced cancer was carried out in 1996 with promising results and acceptable toxicity with a dose rate of 50 μg/kg/min [86]. A phase II trial was later carried out on patients with non-small cell lung cancer [87]. Agteresch et al. [88] 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 U.S. 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 a later trial by this group, beneficial effects of ATP on nutritional status in advanced lung cancer patients were reported [89]. A recent review discusses the use of kinase inhibitors, which interact with ATP binding sites, in anti-cancer therapeutics [90].

In keeping with murine models, ATP treatment has been shown to maintain body weight and decrease cancer cachexia in human studies [91]. In the murine cancer models, intraperitoneal ATP inhibited weight loss in the animals with advanced tumour 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 [92]. In their trial, Agteresch et al. [91] 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. [93] 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 multi-centre, double-blind, randomized control trial will focus on the effects of ATP on survival, tumour response, nutritional status and quality of life [94]. It has been claimed that intravenous ATP infusions can be safely administered to preterminal cancer patients in the home setting [95–97].

Protective effects of ATP against radiation-induced injury in human blood were reported [98]. Ionizing γ-irradiation is a well-known carcinogen capable of inducing tumours, especially in children, even though radiation is commonly used in cancer treatment protocols. Recent papers suggest that γ-irradiation leads to release of ATP, probably via connexin 43 hemichannels and/or P2X7 receptors, which then acts via activation of $P2Y_6$ and $P2Y_{12}$ receptors to mediate repair of DNA damage [99–102].

Breast cancer

Breast cancer is the most common malignant tumour in women and a major health problem worldwide. There is a great emphasis on early diagnosis, but more efficacious therapies are in urgent demand. Growth inhibition of human breast cancer cells by exogenous ATP was first shown in 1993, and it was claimed that the growth arrest was mainly due to elongation of the S-phase of the cell cycle [103]. Chemotherapeutic release of ATP from murine breast tumour cells enhanced tumour regression via apoptosis [104]. The agonist potencies of nucleotides on MCF-7 BCC were shown to be uridine 5′-triphosphate (UTP) ≥ ATP > adenosine 5′-diphosphate (ADP) [105], suggesting that $P2Y_2$ and/or $P2Y_4$ receptors were involved. This was later demonstrated with RT-PCR in MCF-7 breast tumour cells and ATP-activated $P2Y_2$ receptor-linked Ca^{2+} signalling was shown to induce a proliferative response [106]. Extracellular nucleotides co-operate with growth factor to activate c-fos gene expression linked to the proliferative response of MCF-7 cells through activation of $P2Y_2$ receptors [107]. It has been suggested that oestrogen, via ERα receptors, promotes proliferation of breast cancer cells by down-regulating $P2Y_2$ receptor expression and attenuating $P2Y_2$ receptor-induced increase in $[Ca^{2+}]_i$ [108]. Expression of CD73 is negatively regulated by oestrogen acting via the ERα receptor and its generation of adenosine may relate to breast cancer progression [109]. Tamoxifen is used for adjuvant treatment of breast cancer because it prevents growth of cancer cells due to a range of effects in addition to blocking oestrogen actions. Hydrolysis of adenine nucleotides is modified in platelets from breast cancer patients taking tamoxifen [110]. ATP depletion due to hypoxia enhances tamoxifen anti-proliferative effects in T47D breast carcinoma cells [111]. Radioiodide therapy has been used against breast cancer and the iodide symporter gene is expressed in breast tumours. ATP

and UTP, probably via $P2Y_2$ receptors, stimulate sodium/iodide symporter-mediated iodide transport in breast cancer cells [112].

There is much interest in K^+ transport in human breast cancer cells, with the strong possibility that alterations in K^+ ion transport may regulate tumour cell proliferation and apoptosis (see [113]). ATP has been shown to increase K^+ efflux from cultured human breast cancer cells [114]. Thus, it would not be surprising that apoptosis was activated given the profound caspase-3 stimulatory activity of K^+ depletion [115].

Bioluminescence assay of ATP levels in breast tumours has been proposed to detect levels of cell proliferation and hence can be used as a marker for the biological aggressiveness and metastatic potential of breast carcinoma [116]. It has been suggested that over-expression of ATP synthase α-subunit may be involved in the progression of metastasis of breast cancer, representing a potential biomarker for diagnosis, prognosis and therapeutic target for breast cancer [117]. An ATP-based chemotherapy response assay was developed for predicting cell viability [40] and for predicting responses to chemotherapy [118–120].

The Walker 256 rat tumour cell line, which initially arose spontaneously in the mammary gland of a pregnant albino rat, has been used for studies of cancer pathophysiology. Ecto-NTPDases 2 and 5 and CD73 have been identified in Walker 256 tumour cells and are likely to be important in reducing the ratio of ATP/adenosine involved in tumour growth [121, 122]. In a later paper, nucleotide pyrophosphatase/phosphodiesterase (NPP3) was also identified in Walker 256 tumours [123].

MDA-MB-4355 human breast cancer cells secrete nucleoside diphosphate kinase (NDPK) that supports metastases and evidence has been presented to support the notion that secreted NDPK mediates angiogenesis via $P2Y_1$ receptors and suggests that inhibitors of NDPK may be useful as therapeutics [124, 125]. Mitogen-activated protein kinase (MAPK) signalling pathways have been implicated in the regulation of cell proliferation and differentiation. ATP, acting via $P2Y_2$ and/or $P2Y_4$ receptors, activates MAPKs and the P13K/Akt signalling pathway in breast cancer MCF-7 cells [126, 127]. A study of human breast adenocarcinoma, MDA-MB-231 cells, suggests that cell surface ATPase plays important roles in tumour cell migration, drug resistance and the anti-tumour immunoresponse [128, 129]. CD73 facilitates the adhesion, migration and invasion of human breast carcinoma T-47D and MB-MDA-231 cell lines via generation of adenosine [130]. Anti-CD73 antibody therapy inhibits breast tumour growth and metastasis [129]. Bisphosphonates are effective inhibitors of breast cancer as well as for the treatment of metastatic bone disease in women with bone cancer and myeloma [131]. A_3 receptor agonists inhibited the growth of breast tumour-derived bone metastasis, raising the possibility of a therapeutic approach to bone-residing breast cancer [132]. The bisphosphonate, zoledronic acid, had a strong anti-tumour effect, measured by the ATP tumour chemosensitivity assay, on primary breast cancer cells in vitro, which was claimed to be equal or superior to commonly used chemotherapeutic regimens for treating breast cancer [133] as did another bisphosphonate, 5-FdU-alendronate [134].

Proteomic analysis of human breast carcinoma showed that ATP synthase was upregulated in tumours and aurovertin B, an ATP synthase inhibitor, was shown to inhibit proliferation of several breast cancer cell lines [135]. It has been suggested that malignant breast carcinoma cells release ATP that makes a pre-metastatic environment suitable for micro-metastasis in lymph nodes and its nearest afferent lymph vessels [136]. Evidence has been presented that blockade of the action of nucleotides in the context of newly diagnosed breast cancer may provide a useful adjunct to current anti-angiogenesis treatment [137].

ATP enhances epidermal growth factor (EGF) activation of c-fos in Hs578T and T47D breast cancer cell lines, c-fos being an immediate early gene and proto-oncogene that plays a role in cell proliferation, differentiation and apoptosis; the combination of ATP and EGF was anti-proliferative and had strong effects on apoptosis and therefore survival of breast cancer cells [138]. Modulation of ATP-induced calcium signalling by progesterone in T47D-Y breast cancer cells has been reported [139]. ATP induced increase in $[Ca^{2+}]_i$ and actin cytoskeleton disaggregation via P2X receptors in the rat mammary tumour cell line, WRK-1 [140]. ATP increased $[Ca^{2+}]_i$ in breast tumour cells and high concentrations produced apoptosis [141], in retrospect probably via P2X7 receptors. P2X7 receptor-mediated activation of the human breast cancer cell line, MDA-MB-435s, resulted in neurite-like cellular prolongations, an increase in cell migration and the development of metastases, suggesting a potential therapeutic role for P2X7 receptor antagonists [54].

The role of hypoxia in regulating tumour progression is controversial. However, in MCF-7 and MDA-MB-231 breast carcinoma cell lines (as well as the HeLa cervical cancer cell line), the expression of P2X7 receptors is increased by hypoxia and they respond to the P2X7 receptor agonist, 2′(3′)-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate (BzATP), by activating extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), to cause nuclear translocation of nuclear factor-κB [142]. The authors showed further that hypoxia-driven increase in P2X7 receptors enhances invasion and migration of tumour cells. Changes in purinergic signalling during EGF-induced epithelial mesenchymal transition in MDA-MB-468 breast cancer cells have been reported [143]. There was an alteration in the calcium signalling response to ATP, an increase in expression of P2X5 receptor mRNA and a decrease in $P2Y_{13}$ receptor mRNA. Further, it was shown that silencing of P2X5 receptors, which inhibited cell proliferation, led to a significant reduction in EGF-induced vimentin protein expression and it

was suggested that this may represent a novel mechanism for targeting cancer metastasis. Elevated release of ATP in cystic fibrosis is associated with inhibition of breast cancer growth [144].

A_1, A_{2B} and A_3 receptor mRNA has been identified in MCF-7 cells with A_1 receptor agonists leading to MAPK activation [145]. A_1 and A_3 receptor mRNA was shown to be expressed by human breast tumours [146]. Adenosine promotes tumour cell migration and proliferation of MCF-7 and T-47D breast carcinoma cell lines [147]. However, the A_3 receptor-selective agonist, N^6-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide (IB-MECA), down-regulates oestrogen receptor α and suppresses human breast cancer cell proliferation [148, 149]. Adenosine reduces apoptosis in oestrogen receptor-positive (MCF-7 cells) and oestrogen receptor-negative (MDA-MB-468 cells) human breast cancer cells [150]. RNA interference targeting of A_1 receptors, which are upregulated in breast cancer (MDA-MB-468) cells, leads to diminished rates of cell proliferation and induction of apoptosis [151]. The human breast cancer cell line, MDA-MB-231, expresses A_{2B} receptors, which probably mediate cell proliferation [152] and A_{2B} receptor blockade has been shown to slow growth of breast tumours [153]. Tenascin C is expressed in invasive solid tumours, although its role is obscure. Tenascin C has been shown to interact with CD73 to regulate adenosine generation in MDA-MB-231 breast cancer cells [154]. Assessment of adenosine deaminase (ADA) and its isoenzymes ADA1 and ADA2 has been proposed as a reliable test for differential diagnosis of benign and malignant breast disease [155].

Ehrlich ascites tumour cells appeared originally as a spontaneous breast carcinoma in mice and have been widely studied. An early paper showed that these tumour cells did not show a deficit of ATP during growth and concluded that there was no clear relationship between ATP supply and tumour growth [156]. However, later it was shown that extracellular ATP increased $[Ca^{2+}]_i$ [27, 157] and had a growth inhibitory effect on Ehrlich tumour cells [158, 159]. It was shown that ATP elicits changes in phosphoinositide metabolism in Ehrlich ascites tumour cells similar to those produced by a wide variety of Ca^{2+}-mobilizing hormones and growth factors [160]. ATP-induced tumour growth inhibition in Ehrlich ascites tumour-bearing mice was accompanied by a selective decrease in the content of the tripeptide glutathione within the cancer cells in vivo [161]. UTP as well as ATP activated release of Ca^{2+} from inositol triphosphate ($InsP_3$)-sensitive stores in Ehrlich cells [162], suggesting that $P2Y_2$ and/or $P2Y_4$ receptors were involved. Mechanical stress results in the release of ATP from Ehrlich ascites tumour cells, which in turn stimulates both $P2Y_1$ and $P2Y_2$ receptors [163]. A calmodulin inhibitor induced short-term Ca^{2+} entry and a pulse-like secretion of ATP in Ehrlich ascites tumour cells [164]. It was suggested that the increased sensitivity of Ehrlich ascites tumour cells to ATP during the course of tumour growth may be associated with a decrease in ecto-ATPase activity [165].

Prostate cancer

Prostate cancer is the second most common cancer in males and the third leading cause of cancer death. Surgery is the treatment of choice, but post-surgery medical treatment is routinely given. Prostate cancer cells are sensitive to extracellular ATP. Fang et al. [166] first demonstrated that ATP could inhibit the growth of commercially available human hormone-refractory (androgen-independent) prostate cancer PC-3 cells and suggested that this effect was likely to be mediated by P2 receptors. Later, this was shown in DU145 as well as PC-3 prostate cancer cell lines [167, 168]. The potency order of UTP ≥ ATP > adenosine-5′-(γ-thio)-triphosphate (ATPγS) > inosine 5′-triphosphate > uridine diphosphate (UDP) ≥ ADP on human prostate PC-3 cancer cells [169] suggests the presence of $P2Y_2$ and/or $P2Y_4$ receptors. However, $P2Y_1$ receptors were later identified on PC3 cells [170] and a more recent paper claims that activation of $P2Y_1$ receptors, identified by RT-PCR, Western blots and pharmacology, induced apoptosis and inhibited proliferation of these cells [171]. ATP is a potent growth inhibitor of tumours and it was suggested that P2X7 receptors mediate cell death in prostate cancer [172]. Northern blotting showed that both PC-3 and DU145 prostate tumour cells expressed $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ receptors, and after breakdown of ATP to adenosine, there was A_2 receptor activation [173]. It was also shown in this study using RT-PCR that these tumour cells also expressed P2X4 and P2X5 receptors in the DU145 cells and P2X4, P2X5 and P2X7 in the PC-3 cells. ATP inhibited the growth of the tumour cells, but this effect was not mimicked by UTP or adenosine, but BzATP caused an increase in apoptosis in PC-3 cells, probably via P2X7 receptors. Multicellular prostate tumour spheroids prepared from the DU145 prostate cancer cell line were exposed to direct current electrical fields, resulting in ATP release, which activated purinergic receptors to elicit a Ca^{2+} wave leading to stimulation of tumour growth [174]. ATP-induced inhibition of growth of prostate cancer DU145 cells (as well as lung cancer (A549) and pancreatic cancer (Panc-1) cells) via P2X7 receptors was dependent on the P13 kinase pathway that regulates apoptosis and cell growth [175]. $P2Y_2$ receptors mediated resistance to ursolic acid-induced apoptosis in DU145 cells [176]. P2Y receptor agonists stimulated PC-3 prostate cancer cell invasion, via their down-stream ERK1/2 and p38 protein kinases [177]. Both mechanical and hypotonic stress leads to ATP release from DU145 prostate cancer cells [178]. Calcium waves were elicited by mechanical strain releasing ATP from DU145 cancer cells and purinergic receptor activation [179]. ATP enhances the motility and invasion of prostate cancer cells by activating Rho GTPases Racl and

Cdc42 and upregulates the expression of matrix metalloproteinases [180]. A recent study has shown that CD73-deficient mice are resistant to prostate carcinogenesis and concluded that CD73 promotes de novo prostate tumorigenesis and further that anti-CD73 monoclonal antibodies can significantly reduce prostate tumour growth and metastasis [181].

Studies from our own laboratories compared hormone refractory prostate cancer cell lines (PC-3 and DU145) with commercially available normal prostate cells (PNT-2) [182]. 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 % inhibition of cell growth, after ATP 0.1 mM) and more responsive to the mitogenic effects of UTP. The order of agonist potency also differed from HRPC cells, raising the possibility that the control of growth in normal and cancerous prostate cells is different. This 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 P2X5 and/or $P2Y_{11}$ receptors in DU145 cells. The absence of $P2Y_{11}$ receptor mRNA in PC-3 cells made the P2X5 receptor the most likely receptor involved in this cell line.

The discovery of P2X7 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 P2X7 receptor-mediated cell membrane pore formation (using lucifer yellow stain) failed to demonstrate a functional role for these receptors. Coupled with the presence of P2X7 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, this left the explanation of the exact functional role of this receptor subtype uncertain.

The lack of effect of KN-62 at a human P2X7 receptor has been reported previously, where it failed to block permeability lesions to Ca^{2+} and Ba^{2+} and subsequent cytotoxic pore formation [183]. There are known to be many polymorphisms of the P2X7 receptor [184, 185], 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.

There is a differential expression of P2X7 receptors in patients with normal prostates compared to those with prostate cancer. Slater et al. [186] found expression of P2X7 cytolytic purinergic receptors in all 116 pathology specimens of prostate cancer, irrespective of Gleason grade or patient age. P2X7 receptors were also found in normal epithelial cells adjacent to tumour 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 the P2X7 receptor remains largely unknown. In a later paper, it was shown that P2X7 receptor expression in the glandular epithelium is a marker for early prostate cancer and correlates with increasing levels of prostate-specific serum antigen [187].

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 [188]. This response to androgen ablation is lost in hormone refractory cells. However, studies by Martikainen et al. [189] 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 [166, 182], and this has been proposed as a possible mechanism for ATP-induced cell death. ATP, acting at P2Y receptors, 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. ATP and UTP were 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 numbers, whereas ATP induces cell death [182]). 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. [168] 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. ATP release from erythrocytes is increased in blood samples from prostate cancer patients receiving radiation therapy, which would contribute to its beneficial effects, since ATP is a potent inhibitor of tumour growth [190]. A more recent paper claims that activation of $P2Y_1$ receptors induced cell death and inhibited growth of prostate cancer PC-3 cells and it was suggested that $P2Y_1$ receptor agonists may be a promising therapeutic strategy for prostate cancer [171].

Vanoverberghe et al. [168] hypothesized that decreases in the intracellular Ca^{2+} pool were 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, while both 1 nM thapsigargin and 100 μM ATP reduced the Ca^{2+} pool content to a similar extent, only thapsigargin alone had no growth inhibitory effects [168]. As thapsigargin and ATP work on the ER in different ways to lower the Ca^{2+} pool (sarcoplasmic reticulum/ER Ca^{2+} ATPase pump inhibitor vs. $InsP_3$ 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 is associated with prevention of apoptosis and is a common finding in cancer cells. Miyake et al. [191] 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 for 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 growth 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) [32]. 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 tumour cells retained their original phenotype and cellular characteristics. The endothelium has an important role in the regulation of malignant tumour growth [192]. It has been shown recently that secretion of soluble vascular endothelial growth factor (VEGF) receptor-2 by microvascular endothelial cells from human benign prostate cancer is increased by ATP [193]. 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 tumour metastases. An orthotopic model of prostate cancer would add to our understanding of this process and the potential effect of ATP (see section on 'Bone Cancer').

Proliferation of prostate tumour cells is inhibited by adenosine, whereas normal cells are stimulated by adenosine. 2-Chloroadenosine (2-ClAdo) has cytotoxic effects on PC-3 prostate tumour cells, probably by entry into the S-phase of the cell cycle and the induction of DNA strand breaks [194]. It has been claimed that 2-ClAdo induces apoptosis of PC-3 prostate cancer cells [195]. A_3 receptor activity by IB-MECA inhibited prostate cancer cell proliferation and induced cell cycle arrest and apoptosis [196, 197]. Activation of A_3 receptors also suppressed prostate cancer metastasis by inhibiting nicotinamide adenine dinucleotide phosphate oxidase activity [198].

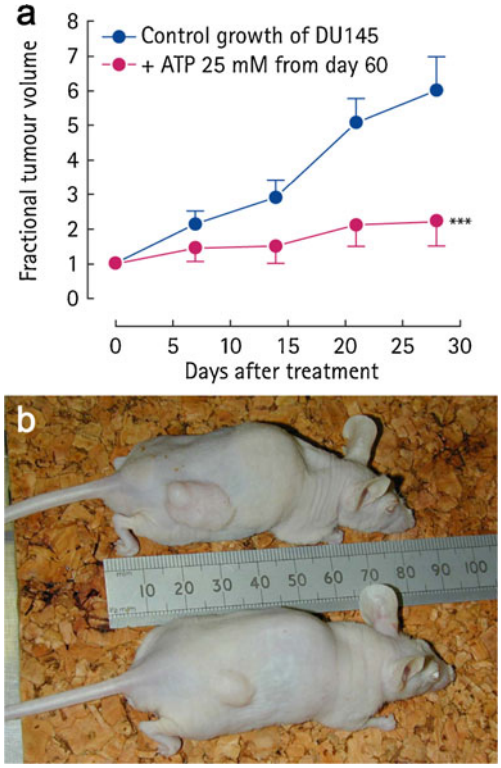

Fig. 2 **a** Effect of ATP (1 ml of 25 mM i.p.) on the fractional growth of HRPC DU145 tumour cells in vivo after 60 days initial growth and **b** effect of ATP (1 ml of 25 mM i.p.) on the growth of implanted DU145 tumour cells in vivo after 60 days of initial growth; the lower mouse received ATP treatment vs. no treatment in the upper mouse. (Reproduced from [32] with permission.)

Colorectal, gastric, oesophageal and neuroendocrine cancer

Colorectal cancer is widespread, but cancer of the oesophagus and gastric and neuroendocrine tumours also occur. Exposure of two colonic adenocarcinoma cell lines, HT-29 and SW-620, to ATP resulted in substantial inhibition of cell growth [199].

Colorectal tumours

ATP and ADP increased $[Ca^{2+}]_i$ in the HT-29 human colonic adenoma cell line [200]. HT-29 cells were depolarised by UTP > ATP > ADP > adenosine [201]. There is a report that cultured human tumour cells derived from the colon (LoVo) are resistant to ATP cytotoxicity, but exposure to verapamil increases sensitivity to ATP [202]. HT-29 cells express P2U (i.e. $P2Y_2$ and/or $P2Y_4$) receptors [203]. Both P2 and neurotensin receptors are expressed by HT-29 cells and both

increase extracellular acidification, but there was no obvious interaction between the actions of these receptors [204]. HT-29-C116E is a highly differentiated sub-clone of the HT-29 colonic cancer cell line and ATP transiently increased Cl^- conductance in these cells [205, 206]. ATP activation of Cl^- conductance was also shown in the T84 human colonic adenocarcinoma cell line [207]. In a later paper, HT-29 cells showed a decrease of intracellular Cl^- and Na^+ and an increase in Ca^{2+} in response to both ATP and UTP via P2U ($P2Y_2$ and/or $P2Y_4$) receptors [208]. RT-PCR studies confirmed the presence of P2U mRNA in both primary cultures of human colorectal carcinoma cells and HT-29 cells and it was suggested that they play a role in the regulation of cell proliferation and apoptosis [209]. $P2Y_2$ receptors mediated resistance to ursolic acid-induced apoptosis in HT-29 cells [176].

Extracellular ATP induced apoptosis and inhibited growth of primary cultures of colorectal carcinomas [209], perhaps via $P2Y_2$ receptors [210] or by an unidentified ATP receptor, mediating actions on the S-phase of cell cycle by inhibiting protein kinase C [211]. Purinergic responses of HT-29 cells are mediated by G protein α-subunits after activation of P2U receptors [212]. mRNA for P2Y receptor subtypes $P2Y_2$, $P2Y_4$ and $P2Y_6$, acting through MAPK cascades, was located on the apical membranes of human colonic Caco-2 adenocarcinoma cells [213, 214]. Caveolin-1 facilitates the hypotonicity-induced release of ATP from basolateral, but not apical, membranes of Caco-2 cells [215]. Using microphysiometry, to measure extracellular acidification rate, $P2Y_2$ receptors were identified on HT-29 colonic carcinoma cells [216], and in a later study, this group showed that both $P2Y_2$ and $P2Y_4$ receptors were upregulated in human colon cancer [217]. Regulation of increase in $[Ca^{2+}]_i$ during $P2Y_2$ receptor activation is mediated by G$\beta\gamma$-subunits [218]. It has been claimed that $P2Y_2$ receptors have oncogenic potential mediating transformation of colorectal RKO cancer cells [219]. ATP induces proliferation of Caco-2 cells via P2Y receptors [220]. Tissue samples from patients with colorectal cancers showed increased expression of an ATP-binding cassette superfamily transporter, multidrug resistance protein-2 [221].

Modulatory effects of the ectonucleotidase CD39 (NTPDase1) on colorectal tumour growth and liver metastasis, and on the expression of both $P2Y_2$ and P2X7 receptors, indicated the involvement of purinergic signalling in these effects [222]. The activity of both CD73 and ADA was markedly higher in primary human colorectal tumours; the ADA level could be correlated with lymph node metastases and histological type, while CD73 activity could be correlated with tumour location and grade [223]. A 40 % increase in ADA activity in human colorectal adenocarcinomas was reported [224]. Dipeptidyl peptidase is a multifunctional cell surface protein which is a binding protein for ADA. Adenosine that is present in increased levels in the hypoxic tumour microenvironment down-regulates the surface expression of this protein in HT-29 cells [225]. RT-PCR showed that gene expression of adenosine kinase is significantly increased in human colorectal cancer [226].

Heterogeneity of chemosensitivity of colorectal adenocarcinoma was determined by a modified ex vivo ATP-tumour chemosensitivity assay, and it was suggested that this could be used to identify patients who might benefit from specific chemotherapeutic agents alone or in combination [227–229]. For years, surgeons have washed the abdominal cavity with distilled water to lyse colorectal cancer cells left after surgery. A study has shown that water induces autocrine release of ATP from epithelial cells, which then causes cell death of tumour cells via P2X7 receptors [230].

Adenosine accumulates in solid tumours and stimulates tumour growth and angiogenesis, while imparting tumour resistance to the immune system, thereby facilitating tumour survival [22, 231]. Adenosine promotes cell proliferation in poorly differentiated HT-29 cells via A_1 receptors; cell growth inhibition was observed in the presence of ADA and A_1 receptor antagonists [232]. In contrast, adenosine had less effect on more differentiated cells with lower proliferation rates (e.g. Caco-2, DLD-1 and SW 403 cell lines) [233], but was still found to stimulate proliferation of such cells (including the colorectal carcinoma human cell lines T84, HRT-18, Caco-2, Colo 320 HSR and MCA-38, the murine liver-derived colon carcinoma cell line) at concentrations present within the tumour extracellular environment; RT-PCR showed that all four P1 (adenosine) receptor subtypes were expressed in all the human carcinoma cell lines studied, but it was speculated that A_{2B} receptors might make a major contribution [234]. A more recent paper claims that adenosine suppresses growth of CW2 human colonic cancer cells by inducing apoptosis via A_1 receptors [235]. There is enhanced A_{2B} receptor expression in proliferating colorectal cancer cells, suggesting that A_{2B} receptor antagonists may be a promising target for colorectal cancer therapy [236].

A single low level intravenous dose of [^{32}P]ATP significantly inhibited the growth of established xenografted subcutaneous human colon adenocarcinoma cell line, HCT116, in nude mice [237]. 8-ClAdo inhibited growth of colorectal cancer cell lines HCT116 and 80514 in vitro and in vivo [238]. Inhibition of primary colon carcinoma growth was elicited by A_3 receptor agonists [239, 240]. However, subsequent papers claimed that A_3 receptors mediate a tonic proliferative effect on Caco-2, DLD1 and HT-29 colorectal tumour cell lines [241]. A phase II, multi-centre study showed that an A_3 receptor agonist (CF101) stabilized the tumour in 35 % of the patients with refractory metastatic colorectal cancer [242]. Elevated expression of A_3 receptors was shown in human colorectal cancer and it was suggested that this could be used as a diagnostic marker and a therapeutic target for colon cancer [243]. 2′-Deoxyadenosine caused apoptotic cell death in the human colon carcinoma cell line, LoVo [244]. Adenosine has also been claimed to induce apoptosis in Caco-2 colonic cancer cell [245].

The chemokine receptor, CXCR4, plays a crucial role in determining the ability of cancer cells to metastasize from the primary tumour. Adenosine upregulates CXCR4 and enhances the proliferative and migratory responses of HT-29 cells [246]. Adenosine down-regulates the cell surface protein CD26, which binds to ADA, on HT-29 colorectal carcinoma cells, thereby facilitating tumour survival [247]. Evidence has been presented that adenosine can stimulate migration of colon cancer cells and that caffeine significantly inhibits this effect [248].

Gastric cancer

The human gastric signet ring cell carcinoma cell line (JR-1) responds to ATP with hyperpolarisation, probably mediated by P2Y receptors [249]. ATP and adenosine reduced proliferation and induced apoptosis in the human gastric carcinoma cell line (HGC-27) [250, 251]. An ATP-based chemotherapy response assay has been used to predict and enhance the benefits of chemotherapeutic drugs in patients with gastric cancer [252, 253]. *Helicobacter pylori* infection of the gastric body contributes to the progression of gastric carcinoma, perhaps by regulation of H,K-ATPase [254]. RT-PCR showed that gastric cancer cells showed a loss of A_3 receptors [255].

Oesophageal cancer

The human oesophageal squamous carcinoma cell line, Kyse-140, and primary cancer cell cultures from patients expressed $P2Y_2$ receptors, which mediated inhibition of growth [26]. A marked heterogeneity of chemosensitivity in oesophageal cancer has been described using the ATP-tumour chemosensitivity assay [256].

Neuroendocrine tumours of the gastrointestinal tract

Neuroendocrine tumours are a heterogeneous group of neoplasms originating from enteric chromaffin cells. RT-PCR showed that these tumours express A_{2A} and A_{2B} receptors and their activation leads to increased proliferation [257], suggesting that they are potential targets for therapy [62].

Biliary cancer

$P2Y_2$ receptors have been identified in human biliary epithelial cancer cells (Mz-Cha-1) [258].

Lung cancer

Lung cancer is the most common cancer in terms of incidence and mortality in the developed world. In males, it is undisputedly the most frequent malignant tumour, but the incidence in females is also rising rapidly. A549 human lung epithelial-like adenocarcinoma cells express P2U (i.e. $P2Y_2$ and/or $P2Y_4$) receptors, which when occupied lead to an increase in $[Ca^{2+}]_i$ [259] which does not inhibit forskolin-evoked cyclic adenosine monophosphate (cAMP) accumulation in these cells [260]. Calcium-dependent release of ATP and UTP (with subsequent increase in adenosine levels) from A549 cells has been reported [261].

A phase II study of intravenous ATP in patients with previously untreated non-small cell lung cancer led to the authors concluding that ATP, at least at the dose and administrative schedule employed, was an inactive agent in patients with advanced non-small cell lung cancer [87].

Erythromycin is widely used in the treatment of respiratory tract infections. It has also been shown to selectively inhibit Ca^{2+} influx induced through P2X4 receptor activation of A549 human lung tumour cells [262]. In this study, it was also shown with RT-PCR that A549 cells express $P2Y_2$, $P2Y_4$ and $P2Y_6$, as well as P2X4 receptors. Transforming growth factor β1 augments ATP-induced Ca^{2+} mobilization, which leads to an acceleration of migration of A549 cells, but it markedly reduces endogenous ATP release [263].

Cachexia is a common feature of lung cancer patients and is associated with metabolic alterations, including elevated lipolysis, proteolysis and gluconeogenesis. An increase in glucose turnover during high-dose ATP infusion in patients with advanced non-small cell lung cancer occurs, perhaps contributing to the reported beneficial effects of ATP on body weight in patients with advance lung cancer [88]. Later randomized clinical trials led to the conclusion that ATP has beneficial effects on weight, muscle strength and quality of life in patients with advanced non-small cell lung cancer as well as enhancing median survival from 3.5 to 9.3 months [89, 97, 264, 265]. ATP infusion restores hepatic energy levels in patients with advanced lung cancer, especially in weight-losing patients [266]. ATP has been claimed to reduce radiation-induced damage [98] and clinical trials are underway to assess the effect of concurrent ATP and radiotherapy treatment on outcome in non-small cell lung cancer patients [94].

ATP induced a significant dose-dependent growth inhibition of five different cell lines: human large cell lung carcinoma (H460), human papillary lung adenocarcinoma (H441), human squamous cell lung carcinoma (H520), human small cell lung carcinoma (GLC4) and human mesothelioma (MERO82) [93]. ATP also had cytotoxic effects on the PC14 lung adenocarcinoma cell line and further enhanced the anti-tumour effect of etoposide (VP16) in both PC14 and the A549 cell line, a human alveolar epithelial cell carcinoma [267]. ATPγS regulated the production of cyclooxygenase-2 and synthesis of prostaglandin E_2 in A549 cells [268].

It has been claimed that extracellular ATP, UTP and UDP stimulate proliferation of A549 lung tumour cells via $P2Y_2$

and $P2Y_6$ receptors as well as an ADP-sensitive receptor that was not the $P2Y_1$ subtype [29]. ATP and ADP strongly inhibited proliferation of the human lung adenocarcinoma cell line, LXF-289, via P2Y receptors [269]. ATP-based chemotherapy response assay has been used to guide the outcomes of platinum-based drug chemotherapy for unresectable non-small cell lung cancer [270, 271]. Cisplatin, a platinum complex, is a widely used anti-cancer agent for the treatment of lung cancer. ATP increases the cytotoxicity of cisplatin in a human large cell lung carcinoma cell line (H460) [272, 273]. Blockade of ATP synthase, located on the plasma membrane, suppresses adenocarcinoma growth [274].

It has been suggested that tumour-infiltrating immune cells can benefit the tumour by producing factors that promote angiogenesis and suppress immunity and because adenosine levels are high in tumours. It has been proposed that A_{2B} receptors on host immune cells may participate in these effects and confirmed when A_{2B} receptor knock-out mice exhibited significantly attenuated growth in a Lewis lung carcinoma (LLC) isograft model [275]. Exposure of human lung cancer cell lines A549 and H1299 to 8-ClAdo induced cell arrest at the G_2/M phase and mitotic catastrophe followed by apoptosis [276–278]. 3-Deoxyadenosine (cordycepin) exerted inhibitory effects on the growth of the mouse LLC cell line by stimulating A_3 receptors [279]. The A_3 receptor agonist, thio-Cl-IB-MECA, inhibited cell proliferation through cell cycle arrest and apoptosis of A549 human lung carcinoma cells [280]. Adenosine induced apoptosis via A_3 receptors in A549 cells [281], SBC-3 [282] and Lu-65 [283] human lung cancer cells. Stanniocalcin-1, a secreted pleiotrophic protein, regulates extracellular ATP-induced calcium waves in monolayers of A549 cancer cells by stimulating ATP release [284]. Lung cancer has been reported to alter the hydrolysis of nucleotides and nucleosides by ecto-nucleotidases in platelets [285].

Cisplatin is widely used for the treatment of cancer, including non-small cell lung cancer. Expression of copper-transporting P-type adenosine triphosphatase, which is associated with platinum drug resistance in tumours, is claimed to be a useful chemoresistance marker for cisplatin actions [286].

Nasopharyngeal cancer

Micromolar concentrations of ATP activated a chloride current that led to shrinking of human nasopharyngeal carcinoma cells [287]. In a later study of human nasopharyngeal carcinoma CNE-2Z cells, it was suggested that the volume-sensitive chloride current is activated via P2Y receptors after autocrine/paracrine release of ATP [288].

Liver cancer

Primary liver malignant tumours are almost always carcinomas and can be further subdivided in hepatocarcinoma, bile duct carcinoma (cholangiocarcinoma) and hepatocholangiocarcinoma. Hepatoma cells have been extensively used to investigate ATP effects. ATP increases calcium uptake by rat hepatoma cells [289]. Nucleotide receptors activate cation, potassium and chloride currents in HTC cells from a rat liver tumour line [290]. CD39 knock-down mice show an increased incidence of spontaneous and induced hepatocellular carcinoma [291]. The hepatoma cell line N1S1-67 has been used to study the signal transduction system activated by ATP, probably $P2Y_2$ or $P2Y_4$ subtypes [292]. An increase in intracellular calcium is followed by the opening of Ca^{2+}-activated K^+ channels leading to membrane hyperpolarisation. Direct intra-arterial injection of a potent inhibitor of ATP production has been proposed as a novel therapy for liver cancer [293]. Vesicular exocytosis plays an important role in release of ATP from HTC cells and a Cl^- channel inhibitor can be used to specifically stimulate ATP release through exocytotic mechanisms [294]. Tumour necrosis factor-α (TNFα) was the first cytokine used for cancer therapy. It has been shown that healthy liver cells are transiently protected from TNFα-mediated cell death by fructose-induced ATP depletion, while malignant cells are selectively eliminated through TNFα-induced apoptosis [295, 296]. Chrysophanol, a member of the anthraquinone family that is one of the components of a Chinese herb including rhubarb recommended for the treatment of cancer, induces necrosis of J5 human liver cancer cells via reduction in ATP levels [297]. Curcumin, a herbal extract, has been reported to inhibit the growth of a variety of cancer cells, and a recent paper suggests that it acts by inhibiting ecto-ATPase activity leading to increased extracellular ATP in hepatocellular carcinoma HepG2 cells [298]. Further, ATP induces ATP release from HepG2 cells [299]. The in vivo effects of ATP infusions on rat hepatocarcinomas have been investigated [300].

Inhibition of hepatoma cell growth by adenosine was reported [301]. In vivo experiments show that the A_3 receptor agonist, CF101, causes inhibition of liver metastasis (following colon carcinoma) [302]. Human hepatocellular carcinoma HepG2 cells express high affinity A_1 receptors, which, when occupied, result in decreased adenosine monophosphate (AMP) and erythropoietin production [303]. ATP and adenosine induce cell apoptosis of the human hepatoma cell line Li-7A via the A_3 adenosine receptor [302, 304]. CF102, a selective A_3 receptor agonist, was claimed to have anti-tumour and anti-inflammatory effects on the liver [305] and has been investigated in a clinical trial for patients with hepatocellular carcinoma [306]. A_{2B} receptors are highly expressed in human hepatoma cellular carcinoma [307].

NTPDase1 (CD39) expression on regulatory T cells inhibits the activity of natural killer cells and promotes hepatic metastatic tumour growth in mice [308]. CD39 deletion, resulting in higher concentrations of extracellular nucleotides, promotes the development of both induced and spontaneous autochthonous liver cancer in mice [309]. Liver metastasis from colorectal cancer is a leading cause of cancer-related morbidity. It is claimed that tailored-chemotherapy, based on ATP-chemotherapy response assay, could be effective for the treatment of initially un-resectable colorectal liver metastasis [310]. The upregulation of ATP-binding cassette transporter genes in hepatocellular carcinoma is mediated by cellular microRNAs [311].

Pancreatic cancer

There are only a few reports about purinergic signalling in pancreatic cancer. Adenocarcinoma arising from pancreatic ducts is responsible for more than 90 % of pancreatic cancers and survival is less than 5 % over a 5-year period. Insulinomas are relatively rare and have a much better prognosis. What we know about purinergic signalling in these cancer cells is mostly from cultured cancer cell lines, which are often used as model systems. An early paper by Rapaport et al. [199] showed growth inhibition of two human pancreatic adenocarcinoma cell lines (CAPAN-1 and PANC-1) in soft agar cultures by treatment with low levels of ATP. A later paper showed that dipyridamole, that prevents uptake of adenosine leading to increased extracellular levels, prevented human pancreatic cancer cell-induced hepatic metastasis in nude mice [312]. Insulinoma cell lines are often compared to isolated islets or β-cells in the same studies and similar conclusions have been reached. For example, ATP at low concentrations promotes insulin secretion from the INS-1 insulinoma cell line and rat islets via P2Y receptors, but inhibits insulin release at high concentrations after being metabolised to adenosine [313]. Also in the CAPAN-1 cell line, derived from human pancreatic adenocarcinoma of ductal origin, ATP and UTP applied to the apical membranes decreased cellular pH indicating HCO_3^- secretion, but were inhibitory when applied to the basolateral membranes [314]. CD39 and P2X7, $P2Y_2$ and $P2Y_6$ receptors are significantly increased in biopsies of pancreatic cancer [315]. High levels of mRNA for CD39 significantly correlated with better, long-term survival after tumour resection in patients with pancreatic cancer. It was claimed that extracellular ATP is cytotoxic for pancreatic cancer cells because of its induction of cell cycle arrest at S-phase and cell death by apoptosis [316]. $P2Y_2$ receptors are functionally expressed on human pancreatic cancer cells mediating cell proliferation [317]. Solid pseudopapillary tumours of the pancreas are rare, comprising only 0.3 % of all pancreatic tumours. The in vitro ATP-based chemotherapy response assay has been used effectively for assessing the chemotherapy for these tumours [318].

Bone cancer, osteosarcoma, myeloma and fibrosarcoma

Interest in bone tumours is motivated not only for the treatment of primary tumours, which are relatively rare, but also for the treatment of metastases. Secondary bone metastases arising from prostate and breast cancer are common, but some primary osteosarcomas occur in children. In addition, there is malignant disease of bone marrow (myeloma) and fibrosarcoma, which can arise from bone.

Bone cancer

Bone metastases are radiographically classified as osteoblastic or osteolytic, resulting from imbalances between osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Osteoblastic lesions, characteristic of prostate cancer, are caused by an excess of osteoblast activity leading to abnormal bone formation. In breast cancer, osteolytic lesions are found in 80 % of patients with stage IV metastatic disease [319] and are characterized by increased osteoclast activity and net bone destruction [320]. Breast cancer bone lesions span a spectrum; most are osteolytic, but up to 15 % are osteoblastic or mixed. Bone metastasis can result in significant bone loss, fractures, pain and hypercalcaemia and spinal cord compression. ATP has been reported to inhibit the growth of bone tumour cells (see [10]).

Significant inhibition of bone tumours by an ADPase, APT102, in combination with aspirin has been demonstrated in two experimental models of bone metastases [321]. APT102 is not directly cytotoxic on the tumour cells, but rather acts via platelets, which are known to contribute to the development of metastasis, since cancer cells travel from a primary site to a distant metastatic site co-existing with platelets in thrombi located in organs and the circulatory system [322]. 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 [323]. 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 analogue (ApppI) which is able to induce apoptosis [324]. Further assessment of this phenomenon and its possible interaction with functional P2X7 receptors found on osteoclasts [325] may help further our understanding of ATP treatment and purinergic receptor pathways in prostate cancer and metastases. Expression of cathepsin L, a cysteine protease

associated with cancer metastasis, which activates heparanase, is predominately enhanced in primary bone tumours, such as osteosarcoma, chrondrosarcoma and multiple myeloma, and tumours which preferentially metastasise to bone (i.e. breast and prostate cancer) and in bone metastases [326]. ATP, ADP and adenosine were most effective in stimulating secretion of active heparanase by tumour cells [327]. Further, heparanase secretion was inhibited by antagonists to P2Y receptors, probably the $P2Y_1$ subtype.

Osteosarcoma

This is the most common primary tumour of bone in children and adolescents. It is characterised by poor differentiation and dysregulation of the genes involved in differentiation. P2X5 receptors, which mediate tumour cell differentiation [328], may be involved in this mechanism. Purinergic regulation of cytosolic Ca^{2+} and phosphoinositide metabolism was reported in rat osteosarcoma cells [329, 330] and human osteoblast-like tumour cells [331]. P2U (i.e. $P2Y_2$ and/or $P2Y_4$) receptors have been implicated in this effect [332]. Modulation of $[Ca^{2+}]_i$ and activation of ERK1/2 and P38 MAPK by ATP, acting via $P2Y_2$ receptors, have been described in osteoblast-like osteosarcoma ROS-A 17/2.8 cells [333]. Butyl benzyl phthalate suppresses the ATP-induced cell proliferation in human osteosarcoma HOS cells, perhaps via P2X receptors [334]. Osteosarcoma cell lines SaOs2 and MG63 express P2X7 receptors; however, another osteosarcoma cell, Te85, did not express P2X7 receptors, but rather P2X5 > P2X4 > P2X6 receptor mRNA, showing that the anti-proliferative effect of ATP on these cells was not via P2X7 receptors [335].

Myeloma

Myelomas are a malignancy of plasma cells, e.g. antibody-producing, differentiated B lymphocytes in bone marrow. 8-Aminoadenosine is an effective cytotoxic agent against multiple myelomas [336]. RPMI 8226 multiple myeloma cells express P2X7 receptor mRNA and protein, as well as P2X1, P2X4 and P2X5 mRNA [337]. A_{2A} adenosine and β-2 adrenergic receptors have synergistic anti-proliferative activity in multiple myeloma models [338]. Heat shock protein 90 (HSP90) is over-expressed in multiple myeloma and 8-chloro-adenosine is currently in clinical trials as an enhancer of inhibition HSP90 to treat multiple myeloma [339].

Fibrosarcoma

Fibrosarcoma is a malignant tumour derived from fibrous connective tissue of the bone. In vivo data show that intraperitoneal ATP slows the growth of spontaneous murine fibrosarcomas without adversely affecting bone marrow radiation tolerance [340]. When fibrosarcoma NCTC 2472 cells were co-cultured with nodose neurons, the sensitivity of P2X2/3 and P2X2 receptors to opioid inhibitory control was decreased and it was suggested that this may contribute to the decreased sensitivity of cancer pain to opioids [341]. Cl^- channels play an important role in ATP release from human fibrosarcoma HT-1080 cells; release does not appear to involve hemichannels [342]. However, in recent papers, it was claimed that maxi-anion channels and pannexin 1 hemichannels are separate pathways for swelling-induced ATP release from murine L929 fibrosarcoma cells [343, 344]. Adenosine A_3 receptor activation elicited inhibition of fibrosarcoma G:5:113 cells [345].

The presence of bone metastases is a major cause of pain [346, 347]. The most common primary sites for bone metastases are breast and prostate with incidence rates for either at 70 % followed by lung at 35 % [348]. Up to 83 % of bone cancer pain patients reported pain that is significantly worse on movement [349]. Despite the availability of bisphosphonates to treat bone cancer pain specifically by preventing bone resorption in addition to NSAIDs and opioids, no new pharmacotherapy has merged in over a decade and patients continue to have bone cancer pain undermanaged [349].

A unifying purinergic hypothesis for the initiation of pain was proposed by Burnstock [350]. One component of the hypothesis was that high concentrations of ATP can be released upon damage of the expanding tumours by bone and connective tissue (see also [351]). It would then stimulate P2X3 receptor-expressing nociceptors present in the afferent nerve endings and result in cancer pain. P2X3 and P2X2/3 receptors have been the most studied purinergic receptors for their role in ATP-mediated nociception since they are highly expressed in a selective subpopulation of nonpeptidergic isolectin B_4-positive primary afferents on peripheral and central terminals [352, 353]. Tumour cells contain an abnormally elevated amount of ATP. Spontaneous and evoked release of ATP from cancer cells by mechanical, hypotonic, electrical stimulation and cell swelling has also been demonstrated (see [342]). Upregulation of P2X3 receptors is found on epidermal nerve fibres in models of bone cancer pain [354]. Minodronic acid, which is a third generation of bisphosphonates, was found to exert antagonistic properties on P2X2/3 receptors and showed analgesic effects in non-cancer pain models [355]. Complementary to its inhibition of bone resorption, the compound is proposed to be effective in relieving bone cancer pain. Radiotherapy is effective in relieving bone cancer pain and P2X6 receptors have been implicated in the underlying mechanism [356].

Thyroid cancer

Thyroid cancers are relatively rare and often only found following a post-mortem examination. However, incidence

may vary in different geographical areas, and a steady increase in the incidence has occurred since World War II. P2Y receptors were shown to be expressed on thyroid cancer cells, but the ATP-induced Ca^{2+}-phosphatidylinositol signalling cascade was found to be impaired [357]. ATP released from human thyroid ARO tumour cells controls the intracellular levels of apurinic apyrimidinic endonuclease redox effector factor-1, a protein involved in repair of DNA lesions [358], thereby controlling HSP90 expression via $P2Y_1$ and $P2Y_2$ receptors [359]. In addition, extracellular ATP was shown to trigger release of IL-6 from human thyrocytes [360]. This observation is of particular relevance as IL-6 is a well-known growth factor for thyroid cells. Increased expression and function of P2X7 receptors have been reported in thyroid papillary cancer [361], and a loss of function polymorphism in the P2X7 receptor (1513A>C) was shown to have a strong association with the follicular variant of this thyroid cancer histotype [362]. It has been claimed that the expression of X-linked inhibitor of apoptosis and P2X7 receptors may predict the aggressiveness of papillary thyroid cancer [363]. The tumour suppressor gene PTEN plays an important somatic role in both hereditary and sporadic cancer. ATP regulates PTEN subcellular localisation in thyroid as well as breast and colon carcinomas [364]. Clodronate is a bisphosphonate used to improve survival of breast cancer patients and prevent bone metastasis. Clodronate-induced apoptosis in human papillary thyroid carcinoma is mediated via the P2Y receptor signalling pathway [365].

PKA-independent inhibition of proliferation and induction of apoptosis of human thyroid cancer cells by 8-ClAdo have been reported [366]. A_3 agonists inhibit thyroid cancer cell proliferation, but apparently independently of receptor activation [367]. Enhanced expression of A_1 receptors in human thyroid carcinoma has been reported [368].

Skin cancer

Ultraviolet (UV) light has been implicated in the genesis of several tissues of cutaneous malignancies, including basal cell carcinoma, melanoma and squamous cell carcinoma. The UV-B component has been identified to have the most severe effects and UV-B irradiation was shown to decrease the amount of P2X1 and $P2Y_2$ receptors and destroy P2X7 receptors, possibly contributing to the malignant transformation of keratinocytes [369].

Basal cell and squamous cell carcinomas

Basal cell and squamous cell carcinomas are tumours that usually arise after 50 years of age, squamous cell carcinoma being more frequent and more aggressive than basal cell carcinoma. Local administration of nucleoside analogs inhibited growth of basal cell carcinomas [370]. The A431 human cutaneous squamous cell (epidermal) carcinoma cell line expressed P2 receptors [371], which when occupied led to an increase in $[Ca^{2+}]_i$ [372, 373]. Stimulation of A431 cells by ATP caused production of $InsP_3$ [374], suggesting that P2Y receptors were involved. A mechanism based on the release of ATP, perhaps acting at P2X receptors, was shown to be involved in human lymphokine-activated killing of human carcinoma and melanoma cells [375].

An investigation of purinergic signalling on the non-melanoma skin cancers, basal cell carcinoma and cutaneous squamous cell carcinoma was carried out [28]. Immunohistochemical analysis of both frozen and paraffin sections of these human skin carcinomas showed expression of P2X5, P2X7, $P2Y_2$, $P2Y_2$ and $P2Y_4$ receptors. P2X5 and P2Y receptors were heavily expressed on both basal cell and squamous cell carcinomas, and P2X7 receptors were expressed in the necrotic centre of nodular basal cell carcinomas and in apoptotic cells in superficial multifocal and infiltrative basal cell carcinomas. $P2Y_1$ receptors were only expressed on the stroma surrounding tumours. $P2Y_4$ receptors were found in basal cell, but not squamous cell carcinomas. Functional studies on the A431 squamous carcinoma cell line supported the view that low concentrations of ATP and UTP caused an increase in cell number, whereas high concentrations caused a significant decrease, while the potent P2X7 receptor agonist, BzATP, also caused a significant decrease.

In addition to ATP causing apoptosis of cultured A431 cells via P2X7 receptors, it was shown that UTP and adenosine (following breakdown of ATP) also induced cell death [376]. In in vivo experiments in mice, skin papillomas followed by squamous spindle cell carcinomas induced by local treatment with 7,12-dimethyl-benz(*a*)anthracene (DMBA), followed by tumour promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA) were used to show that application of BzATP, a potent P2X7 receptor agonist, inhibited the formation of DMBA/TPA-induced skin papillomas and carcinomas [377]. At the completion of the study at week 28, the proportion of living animals with cancers in the DMBA/TPA group was 100 % compared to 43 % in the DMBA/TPA + BzATP group. γ-Irradiation, which causes growth arrest and death of tumour cells, induces P2X7 receptor-dependent ATP release from B16 melanoma cells [99]. Skin cancer can be induced by drinking water containing arsenic. A recent paper claimed that arsenic may induce malignancies by reducing calcium release from ER by $P2Y_4$-mediated ATP actions in human primary keratinocytes [378].

It is concluded that $P2Y_2$ receptors mediate proliferation, P2X5 receptors mediate differentiation (and are therefore anti-proliferative) and P2X7 receptors mediate cell death. ADA in saliva has been identified as a diagnostic marker of squamous cell carcinoma of the tongue [379].

Melanoma

Malignant melanoma is an aggressive cancer with a high potential for metastasis that originates from melanocytes, the pigment-producing cells of the skin. ATP inhibited the growth of both animal and human melanoma cells in vivo [18, 380, 381]. Amelanotic hamster melanoma A-Mel3 cells, grown subcutaneously in hamsters, have been used to study ATP levels in relation to blood flow [382, 383]. CD39 is over-expressed in differentiated human melanomas compared to normal melanocytes [384]. Extracellular ATP has growth-inhibiting properties in a highly metastatic liver-colonising murine B16 melanoma cell line in vitro [385].

Increased expression of P2X7 receptors in 80 patients with superficial spreading melanomas was reported [386]. Labelling of P2X7 receptors also extended 2 μm from the melanoma into the keratinocyte layer of the adjacent epidermis. Conversely, P2X1-3 and $P2Y_2$ receptors (found on normal, but not neoplastic skin) were fully de-expressed within 2 μm of the melanoma. A later paper from another group confirmed that human melanomas express functional P2X7 receptors, which produce apoptosis, and it was suggested that they may represent a novel target for melanoma therapy [37]. Overexpression of P2X7 receptors was produced when P2X7 receptor cDNA was transfected into B16 murine melanoma; tumour growth was significantly enhanced in vivo, but not in vitro [387]. A low pH environment (mimicking the hypoxia and acidosis commonly seen in solid tumours) was shown to induce ATP release from B16 melanoma cells to act via P2X7 receptor to increase proliferation and the P2X7 receptor antagonist, oxidised ATP, significantly inhibited tumour growth [388]. Expression of $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptor mRNA and protein in human melanomas was reported [45]. This study also showed that incubation of A375 melanoma cells with the $P2Y_1$ agonist, 2-methylthio ADP, caused a dose-dependent decrease in cell number, while the $P2Y_2$ receptor agonist, UTP, caused an increase in cell numbers. Melanoma is characterised by apoptosis resistance connected to irradiation- and chemo-resistance, and it was claimed that the P2X7 receptor has an anti-apoptotic function in melanoma cells, since ATP activation suppresses induced apoptosis, while with knock-down of P2X7 receptor gene expression, ATP-induced apoptosis was enhanced [389].

Athymic mice, injected with A375 human melanoma cells, were treated daily with intraperitoneal injections of ATP. The in vivo tumour volume and animal weight were measured over the course of the experiment and the final tumour nodule weight was measured at the end of the experiment. Tumour volume decreased by nearly 50 % by 7 weeks in treated mice. Weight loss in untreated animals was prevented by ATP. Histological examination of the excised tumour nodules showed necrosis in the ATP-treated tumours only. The presence of $P2Y_1$ and P2X7 receptors, previously proposed as extracellular targets for melanoma treatment with ATP, was demonstrated in the excised specimens by immunohistochemistry. This paper provides further support for the use of ATP as a treatment for melanoma [38].

ATP released by murine B16 melanoma cells up-regulates the expression of CD39 on tumour-resistant regulatory T cells; the upregulated CD39 degrades ATP to adenosine, which then contributes to the immunosuppressive environment of the tumour [390]. It has been claimed that ATP production by B16 melanoma tumour cells may contribute to recruitment and stimulation of regulatory T cells, resulting in an immunosuppressive environment [391]. They showed further that implanting B16 melanomas into CD73 knock-out mice, which are impaired in adenosine production, led to a significant slowing down of the growth of the tumours.

Adenosine has also been investigated in relation to melanomas. For example, administration of adenosine was shown to potentiate the actions of chemotherapeutic agents in vivo. In mice inoculated with B-16 melanoma cells, adenosine (268 mg/kg) was injected 5 days before administration of cyclophosphamide (50 mg/kg). This combined treatment reduced the number of melanoma foci by 60 %, while the chemotherapy alone only reduced them by 45 %. Moreover, a protective effect of adenosine against chemotherapy-induced decrease in leukocyte counts was seen in this study [392]. Cell motility, an essential component of tumour progression and metastasis, is mediated by adenosine in human melanoma cells, probably via the A_1 receptor subtype and the possibility of anti-metastatic therapies based on inhibition of A_1 receptor activation was raised [393]. A_1, A_{2A}, A_{2B} and A_3 receptor subtypes were all identified in the human malignant melanoma A375 cell line with RT-PCR and pharmacological evidence [394]. Adenosine, arising from released ATP, acts on adenosine receptors that are mediators of both reduction of cell proliferation (probably via A_3 receptors) and promotion of cell death (probably via A_{2A} receptors) of cultured human melanoma A375 cells [395]. A later paper, from another group, confirmed that A_3 receptor activation led to growth inhibition of melanoma cells and showed that this occurs both in vitro and in vivo [396]. A_3 receptor activation inhibits cell proliferation via phosphatidylinositol 3-kinase 1/2 phosphorylation in A375 human melanoma cells [397]. An A_3 receptor agonist, 2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine, produces an effective anti-tumour immune response in melanoma-bearing mice, involving the activation of natural killer cells and T cells [398]. However, a recent report claims that adenosine, acting via A_3 receptors, promoted cell proliferation of human C32 malignant melanoma cells [399]. A review of adenosine receptors and human melanoma was published in 2003 [400]. A_{2B} receptor blockade can impair IL-8 production, which is elevated in patients with malignant melanoma, while blocking A_3 receptors decreases VEGF, which promotes angiogenesis and metastasis of human carcinoma cells [401].

Evidence has been presented to suggest that adenosine could be a potent immunoregulatory factor affecting both cytokine production and cytotoxic activity of anti-melanoma-specific T cells [402]. Serum and peritoneal fluid ADA levels were higher in malignant ovarian neoplasms and it was suggested that this may be a useful biomarker in diagnosis and management of ovarian tumours [403].

It has been proposed that extracellular ATP released by dying tumour cells accumulates in high concentrations that not only act as danger signals in the immune system, but can also directly kill adjacent tumour cells via P2X7 receptors [43]. Using a genetically modified melanoma cell line, they showed that the anti-tumour effect of ATP can be amplified by inhibition of the ectonucleotidase CD39. In another recent paper, it was shown that in a melanoma model, tumour growth is impaired in CD73-deficient mice [42].

Cervical, ovarian and uterine cancer

Cervical cancer

Cervical cancer today can be effectively diagnosed in the very early phases of the disease, thus reducing by three to four times the death rate compared to pre-early diagnosis times. However, due to its high incidence, an efficacious medical treatment, besides surgery, is needed. HeLa cells, derived from human cervical cancer cells, have been widely used in studies of the involvement of purinergic signalling in cancer. Extracellular ATP was shown to activate K^+ movements in HeLa cells [404] and to elevate $[Ca^{2+}]_i$ following interaction with a nucleotide receptor [405]. Activation of $P2Y_2$ receptors with UTP and ATP caused proliferation and inhibited the activity of Na^+/K^+-ATPase in HeLa cells [406]. It has been claimed that $P2Y_6$ and $P2Y_4$ receptor expression on HeLa cells increases during their proliferation [407]. UDP activation of $P2Y_6$ receptors also induced proliferation of HeLa cells, but via a different second messenger pathway from that produced by the $P2Y_2$ receptor [408]. Oestrogen reverses the apoptotic effects mediated by P2X7 receptors in normal cervix, but not in human cervical epithelial cancer cells [30]. The authors suggest that oestrogen may have a permissive effect for the development and growth of cervical cancer. A truncated P2X7 receptor variant (P2X7-j), endogenously expressed in cervical cancer cells, antagonises the full-length P2X7 receptor through hetero-oligomerization [409]. ATP induced Ca^{2+} mobilization and cell proliferation of four different human cervical cancer cell lines via the activation of nuclear factor κB, a transcription factor implicated in regulatory genes involved in neoplastic transcription [410]. The ATP cell viability assay has been recommended for the measurement of intrinsic radiosensitivity in cervical cancer, which shows how well a tumour is responding to radiotherapy [411].

Most human cancers derive from epithelia and the proliferation and differentiation of epithelial cells are crucially dependent on EGF receptor function. Stimulation of $P2Y_1$ receptors on HeLa cells, for as little as 15–10 min, triggers EGF receptor mitogen signalling and $P2Y_1$ antagonists reduced basal cell proliferation [412]. OppA, the ecto-ATPase of *Mycoplasma hominis*, induced ATP release and cell death of HeLa cells [413]. Human connexin 30.2/31.3 mediates enhanced ATP release from HeLa cells [414]. Gentle mechanical stimulation also released ATP from HeLa cells [415].

ADP and AMP hydrolysis, as well as ADA activity, were enhanced in the early (NIC I) stage of cervical intraepithelial neoplasia and uterine invasive cancer [416]. They also showed that the ADA isoform, ADA 1, was present in platelets from neoplastic patients, suggesting platelet participation in tumour development.

Ovarian cancer

Epithelial ovarian tumours represent about 25 % of female organ malignancies and have a higher mortality rate than cervical or uterine cancers. Extracellular ATP raised $[Ca^{2+}]_i$ and stimulated growth of human ovarian carcinoma OVCAR-3 [417] and SKOV-3 cells [418]. ATP enhances the penetration into human ovarian cancer cell lines (OC-109, OC-238 and OC-7-Nu) of adriamycin, a drug used as a cytotoxic agent to reduce tumour progression [419]. It has been suggested that ATP may act as an extracellular messenger in controlling the ovarian epithelial cell cycle through $P2Y_2$ receptors on EFO-21 and EFO-27 human ovarian cancer cells [420]. ATP tumour chemosensitivity assays have been recommended to assess the viability of chemotherapy for the treatment of primary recurrent platinum-resistant epithelial ovarian cancer [421–426]. A combination of zoledronic acid and fluvastatin has been claimed to have activity against ovarian (and breast) cancer based on this assay [427]. A_2 receptor antagonism inhibits angiogenic activity of human ovarian cancer cells [428]. Variants of the RB1 gene have been implicated as risk factors for invasive ovarian cancer [429]. A recent study has shown that the presence of ATP during the treatment of human ovarian carcinoma with cisplatin leads to additive cytotoxicity [430].

Uterine cancer

$P2Y_2$ receptors were claimed to participate in control of the cell cycle and suppression of proliferation of HEC-1A and Ishikawa human endometrial carcinoma cells [431]. Expression of copper-transporting P-type ATPase has been proposed as a prognostic factor for human endometrial carcinoma [432]. The P2X7 receptor has been claimed to be a novel biomarker for uterine epithelial cancers [433]. Tissue levels of P2X7 mRNA and protein differentiate between normal and hyperplastic from

pre-cancerous and cancerous endometrium; there is decreased expression of P2X7 receptors on endometrial epithelium in pre-cancerous and cancer cells [434]. The reduced expression of P2X7 receptors in uterine cancer cells was claimed to be the result of increased expression of micro RNAs that regulate P2X7 expression [435], but the pathophysiological significance of this phenomenon is unclear.

It was shown, using an ATP tumour chemosensitivity assay (see [436]), that topotecan has a significant cytotoxic effect on uterine squamous cancer cell lines A-431, CaSki and C-33, which appeared to be superior to cisplatin [437]. A reduction in ectonucleotide pyrophosphatase/phosphodiesterase and ADA activities in patients with uterine cervix neoplasia have been reported [438].

Leukaemia

Leukaemia consists of a group of malignant diseases that start in the bone marrow and cause overproduction of blood cells that are massively released into the bloodstream. There are four common types of leukaemia: chronic lymphocytic (or lymphoid) leukaemia (CLL), chronic myeloid leukaemia (CML), acute lymphocytic (lymphoblastic) leukaemia and acute myeloid leukaemia (AML). These basic types can further be subdivided into subtypes. In particular, AML can be further subdivided into myeloblastic, promyelocytic, myelo-monocytic, monocytic, megakaryoblastic leukaemia and erythroleukaemia. Uncontrolled proliferation of lymphoid cells can also start in lymphoid organs, with little spill over of neoplastic cells into the blood, until the late stages of the disease. These tumours are classified into Hodgkin's or non-Hodgkin's lymphomas.

Lymphocytic leukaemia

The enzymes concerned with purine degradation, CD73, ADA and purine nucleoside phosphorylase, were measured in the bone marrow or blood of patients with both CML and CLL [439]. The levels of these enzymes varied with the type of leukaemia.

ATP and UTP activated superoxide formation in HL-60 promyelocytic leukaemic cells [440] and ATP also increased $[Ca^{2+}]_i$ in these cells [441, 442] probably via $P2Y_2$ receptors [443, 444]. Reduced proliferation was produced by ATP and UTP in both HL-60 promyelocytic and U937 promonocytic human cell lines [445], perhaps via A_3 receptors [446]. Two different P2Y receptor subtypes were proposed to be responsible for the increase in $[Ca^{2+}]_i$ in HL60 cells, a $P2Y_2$ (or $P2Y_4$) receptor and probably a $P2Y_1$ receptor [447]. ATP enhanced the adherence of HL-60 cells to bovine pulmonary artery endothelial cells [448]. ATP increased cAMP production in undifferentiated HL-60 cells [449] and induced differentiation and suppressed cell growth via an unknown receptor (not $P2Y_2$) [450]. Histamine inhibits ATP-induced rise in $[Ca^{2+}]_i$ through the activation of PKA in HL-60 cells [451]. Adenosine, after breakdown of ATP, contributes to the inhibitory effect of ATP on proliferation of HL-60 cells [25]. It was claimed that ATP-dependent suppression of proliferation was largely via adenosine receptors, while ATP induction of differentiation was via P2X receptors [452]. It was proposed that $P2Y_{11}$ receptors mediated ATP-induced differentiation of both myeloblastic HL-60 and promyelocytic NB4 cells into reactive neutrophil-like cells [57, 453]. RT-PCR analysis showed expression of P2X5, P2X7 and $P2Y_{1\text{-}11}$ receptors (except for $P2Y_6$) mRNA in HL-60 cells during the course of differentiation and CD39 and CD73 were upregulated during maturation [454]. $P2Y_2$ and $P2Y_{11}$ receptors were upregulated during granulocytic differentiation of HL-60 cells [455]. ATP induced apoptotic cell death of both HL-60 and F-36P leukaemia cell lines [456]. Static magnetic field exposure of HL-60 cells increased the increase in $[Ca^{2+}]_i$ produced by ATP [457, 458]. A_3 receptor agonists were shown to inhibit proliferation and induce apoptosis of HL-60 leukaemia cells [459, 460]. ADP and ATP increased $[Ca^{2+}]_i$ in CB1 cells, isolated from a patient with T-acute lymphoblastic leukaemia [461], probably via $P2Y_1$, $P2Y_{12}$ or $P2Y_{13}$ receptors. Apoptotic cells release ATP, which is an early signal to recruit phagocytes. Pannexin 1 channels have been identified that mediate ATP release in Jurkat T lymphoblastoma leukaemia cells [462].

P2X7 receptors have been described in human leukaemic lymphocytes [463, 464]. B cell CLL is one of the most common haemopoietic tumours. Evidence has been presented to suggest that expression and function of P2X7 receptors, which can mediate cell death or proliferation depending on the level of activation, may correlate with the severity of B cell CLL [463]. A 1513C polymorphism of the P2X7 receptor gene has been associated with an increased risk of developing CLL [465], but later studies showed that this might only be relevant in the rare familial form of the disease [466]. P2X7 receptor expression was significantly higher (relative to bone marrow mononuclear cells) in cells from patients with lymphoblastic leukaemia (as well as acute and chronic myelogenous leukaemia) [467]. It has been claimed that the P2X7 receptor-mediated cytotoxic effects on KGla and J6-1 leukaemia cell lines may occur independently of the calcium response [468]. In paediatric acute leukaemia, RT-PCR and Western blots showed that P2X1, P2X4, P2X5 and P2X7 receptors were upregulated, while P2X2, P2X3 and P2X6 receptors were absent or marginally expressed and the highest expression of P2X7 receptors was found in relapsed patients [469]. They also showed a significant decrease in the expression of P2X4, P2X5 and P2X7 receptors after complete remission after chemotherapy.

Adenosine was shown to have cytotoxic effects on mouse leukaemia L1210 cells [470]. In the human leukaemia cell

line, U-937, ATP-induced cytotoxicity was biphasic, the initial response due to ATP, while the later response was due to adenosine, after ectoenzymic breakdown of ATP [471]. A_3 receptors were also identified on Jurkat cells, a human leukaemia cell line [472]. Guanosine and deoxyguanosine are toxic to Jurkat cells through two mechanisms: ATP depletion, causing necrosis, and the accumulation of dGTP, resulting in apoptosis [473]. 2-ClAdo produced cell death of leukaemic B cells [474]. Adenosine was shown to suppress the growth of the human T lymphocyte leukaemic cell line MOLT-4 [475]. A_1 and A_2-like receptors exert opposite effects on 5-hydroxytryptamine release from a mastocyte tumour cell line, rat basophilic leukaemic RBL cells [476].

Lymphomas

Adenosine, acting via A_2 receptors, and CD39 have been suggested as novel targets for augmenting human follicular lymphoma immunotherapy [477]. It has been suggested that increased CD39 expression on $CD4^+$ T lymphocytes has clinical and prognostic value in CLL [478]. CD73-generated extracellular adenosine favoured growth and survival of CLL cells [479]. 8-ClAdo has been evaluated in phase I clinical trials for the treatment of CLL using the mantle cell lymphoma cell lines, Granta 519, JeKo, Mino and SP-53 [480]. 8-ClAdo inhibited the rates of DNA synthesis and depleted ATP resulting in cell death and inhibition of growth. It has been suggested that the ATP-CD39-A_{2A} receptor pathway is one mechanism for T cell hyporesponsiveness in follicular lymphoma [477]. Purine nucleoside analogs, including clofarabine, nelarabine and forodesine, are being explored for the treatment of CLL [481]. A_3 receptors were shown to mediate inhibition of lymphoma cell growth [482]. ADA was immunolocalized on human B cell lymphomas [483]. Extracellular ATP increased cation permeability of CLL lymphocytes [484].

Iron complexed by ATP induces lymphomas in mouse organs [485]. Anaplastic large cell lymphomas are a distinct subset of non-Hodgkin's lymphomas and multikinase inhibitors have been recommended for the treatment of these tumours [486]. Inhibition of the expression and function of P2X7 receptors attenuated the metastatic capability of murine P388D1 lymphoid neoplasm cells [487]. Activation of P2X7 receptors caused depletion of intracellular ATP in T lymphoma cells [488]. Yac lymphoma cells actively secrete ATP in response to P2X7 receptor activation and the ATP amplifies P2X7 receptor signalling or acts on other purinoceptor subtypes to modulate tumour growth and the anti-tumour immune response [489].

Myeloid (myelogenous) leukaemia

Myeloid leukaemias consist of any of the blood cells originating in the blood-forming (myeloid) tissue of the bone marrow and K562 is a leukaemic cell line established from the pleural effusion of a patient with chronic myelogous leukaemia. ADP was shown to be a potent stimulus for calcium mobilization in K562 cells probably acting via P2T (i.e. $P2Y_{12}$) receptors [490]. ATP, UTP, BzATP and adenosine were cytotoxic on K562 cells [491].

A frameshift polymorphism of the P2X5 receptor elicits an allogeneic cytotoxic T lymphocyte response associated with remission of CML [492]. Mouse myelomonocytic leukaemic M1 cells were established from spontaneous myeloid leukaemia mouse strains and ATP was shown to enhance differentiation in these cells [493]. 4-Aminopyridine, a voltage-gated potassium channel blocker, induced apoptosis of human AML cells via increasing $[Ca^{2+}]_i$ through P2X7 receptor pathways [494]. P2X7 receptor activation induces reactive oxygen species formation in murine erythroleukaemia cells and it was suggested that this may be involved in downstream events of P2X7 receptor activation, other than apoptosis, in erythroid cells [495]. P2X7 receptor agonists mediate cation uptake into human myeloid leukaemic KG-1 cells [496]. Adenosine analogues have been proposed as a possible differentiation-inducing agent against AML in B4 cells [497]. ATP depletion triggers AML differentiation (and is therefore anti-proliferative) through an ATR/Chk1 protein-dependent and a p53 protein-independent pathway and therefore is a promising strategy for treatment of AML [498]. AML cells express P2X1, P2X4, P2X5 and P2X7 and all P2Y receptor subtypes [499]. However, in contrast to that observed in normal human leucocytes, P2 receptor stimulation induced a significant inhibition of both proliferation and migration in vitro and engraftment in immunodeficient mice.

Differences were detected in ATP-binding cassette subfamily B member 1 (*ABCB1*) mRNA expression in leukocytes, polymorphonuclear and mononuclear cells in patients with de novo CML [500].

Erythroleukaemia

Extracellular ATP inhibited the growth of murine erythroleukaemia MEL cells [24]. P2X7 receptors mediate cell death and microparticle release in MEL cells [501]. ATP and UTP increased $[Ca^{2+}]_i$ in the HEL human erythroleukaemia cell line [502]. It was later proposed that HEL cells expressed both $P2Y_2$ (and/or $P2Y_4$) and probably $P2Y_1$ receptors [503]. The P2U receptor engages the heterotrimeric G protein G_{16} to mobilize Ca^{2+} in HEL cells [504].

ATP causes lysis of the monocytic leukaemic cell line THP-1, probably via P2Z (i.e. P2X7) receptors [505]. P2X7 receptor-mediated pore formation was described in THP-1 cells [506]. Adenosine, via cAMP, was shown to inhibit differentiation (and therefore increase proliferation) of mouse erythroleukaemic MEL cells [507].

Bladder cancer

Three main types of bladder cancer are described: transitional cell carcinoma (TCC), squamous cell carcinoma and adenocarcinoma. Bladder cancer is more common in industrialized countries; however, it is also frequent where bilharzial (*Schistosoma hematobium*) infections occur (i.e. Egypt). The effect of ATP has been investigated in high grade 3 (G3) superficial TCC of bladder where the tumour is confined to the mucosa or submucosa [33]. Commercially available HT-1376 cells were found to express the same purinergic receptor mRNA as PC-3 prostate cancer cells (P2X4,5,7 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 P2X5 and/or $P2Y_{11}$ receptors in this anti-neoplastic response, although a possible contributory effect of P2X7 receptors could not be discounted. This functional receptor profile and the order of agonist potency were the same as that seen in HRPC cells, although G3 TCC cells were more sensitive to the cytotoxic effects of ATP (reduction of growth by 88.5±4.4 vs. 45±2.3 % for PC-3 cells at ATP 0.1 mM). These 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).

Although studies have demonstrated a potential differentiating role for P2X5 [58, 508] and $P2Y_{11}$ receptors [57], no studies have implicated these receptors in the induction of apoptosis. Apoptosis has classically been linked to the P2X7 receptor, although, despite the presence of P2X7 receptor mRNA, a significant functional role for this receptor subtype could not be elicited. ATP significantly increased apoptosis after 72 h [33]. Ryten et al. [508] demonstrated that the activation of P2X5 receptors mediated the stimulation of cell differentiation markers and thereby inhibited proliferation in skeletal muscle cells. It is therefore possible that activation of P2X5 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 tumour 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 tumours

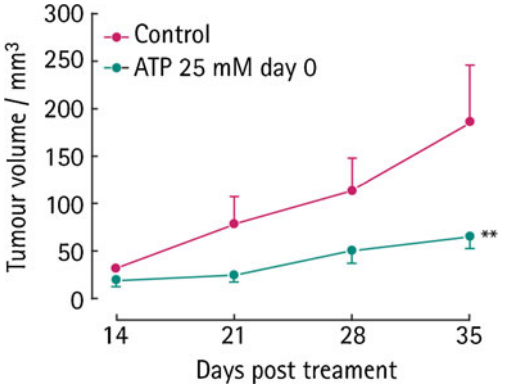

Fig. 3 Effect of daily intraperitoneal ATP (1 ml of 25 mM i.p.) from day 0 on the growth of freshly implanted human bladder TCC HT-1376 tumour cells in vivo. (Reproduced from [33] with permission.)

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 [33]. There is high correlation between adenine nucleotide content and bladder tumour progression [509]. There was upregulation of P2Y receptors in T24, a transitional cell carcinoma cell line [510].

Growth of bladder carcinoma J82 cells was inhibited and apoptosis was induced by adenosine [511]. In the human bladder T24 cell line, adenosine increased $[Ca^{2+}]_i$ and cAMP production as well as IL-8 secretion, via A_{2B} receptors [512]. It has been reported that A_{2B} receptor blockade slows the growth of bladder tumours [153].

A differential pattern of ectonucleotidases in the more malignant human bladder cancer cells compared with cells derived from an early stage of bladder cancer has been described [513].

The distinct advantage with bladder tumours 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. 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-tumour antibiotic mitomycin C significantly increased its effect on cell death, reducing the chemotherapeutic drug concentration at which 50 % of cells were killed by a factor of 10 [33] (Fig. 4a). The same effect was seen with ATP and mitoxantrone, an anti-tumour antibiotic approved for use in the treatment of HRPC [32] (Fig. 4b). However, the cytotoxic effect of these combinations was

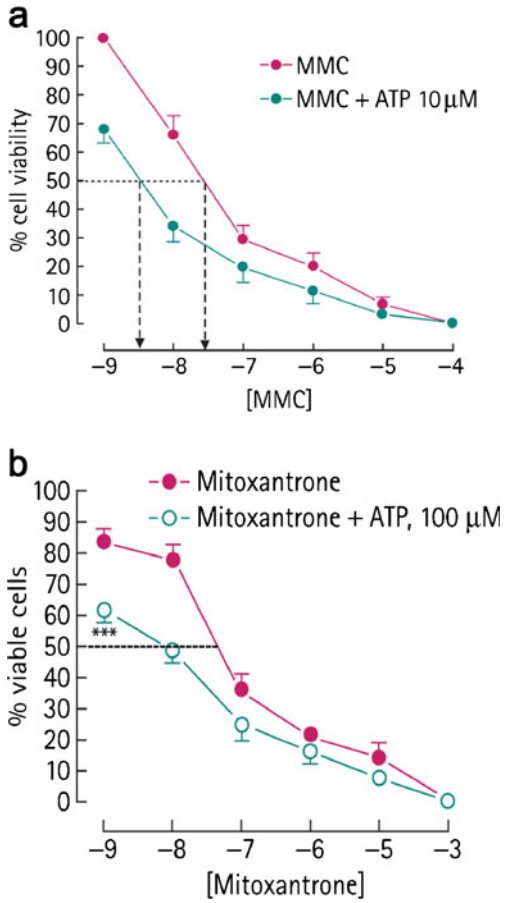

Fig. 4 **a** Dose–response curve of the effect of combining ATP with mitomycin C (*MMC*) vs. MMC alone on the viability of human bladder TCC HT1376 cells in vitro. **b** The effect of combining mitoxantrone and ATP on the viability of HRPC PC-3 cells in vitro. All points are the mean (S.E.M.) unless occluded by the symbol. ***$P<0.001$. (**a** Reproduced from [33] and **b** from [182] with permission.)

additive only and not synergistic. This is probably explained by the respective mechanisms of action. Both anti-tumour 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 [18]. 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 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. Hemorrhagic cystitis is an adverse effect of cancer therapy with cyclophosphamide. Pretreatment of mice with the selective P2X7 receptor antagonist, A438079, reduced the nociceptive behaviour score and virtually abolished the increases in bladder myeloperoxidase activity, an indicator of neutrophil migration, induced by cyclophosphamide [41].

Brain tumours

Neuroblastoma/neuroma

Neuroblastomas are malignant tumours comprised of embryonic nerve cells. They may originate in any part of the sympathetic nervous system, most commonly in the medulla of the adrenal gland and secondary tumours are often widespread in other organs and in bone. Neuromas refer to any tumours derived from cells of the nervous system, often categorised more specifically, e.g. neurofibroma, neurilemmona and reactive neuroma.

Early studies showed that ATP and adenosine stimulated the production of cAMP in the cloned line NS20 of mouse neuroblastoma [514–517]. Later, the mouse neuroblastoma N18TG-2 × rat C6BU-1 glioma hybrid cells, NG108-15, were used to study the effect of nucleotides [518–526], probably via P2U ($P2Y_2$ and/or $P2Y_4$) receptors [527–529]. Later P2Z (= P2X7) receptors were identified on NG108-15 cells [530–534] and maybe also $P2Y_6$ receptors [535]. Subsequently, RT-PCR analysis detected transcripts for both $P2Y_2$ and $P2Y_6$ receptors in NG108-15 cells, but not for $P2Y_1$ or $P2Y_4$ [536]. UDP arrests the cell cycle and induces apoptosis in human neuroblastoma SH-5Y5Y cells over-expressing the $P2Y_6$ receptor [537]. Ecto-alkaline phosphatase was shown to be important for the metabolism of nucleotides by NG108-15 cells [538, 539].

ATP applied to mouse neuroblastoma Neuro-2A cells resulted in a selective enhancement of plasma membrane permeability for Na^+ relative to K^+, but also inward Cl^- pumping [540], probably via P2Y receptors [541]. ATP and UTP were shown to increase $[Ca^{2+}]_i$ in the murine neuroblastoma cell line NIE-115 [542], perhaps via $P2Y_2$ and/or $P2Y_4$ receptors. Later, P2X7 receptor mRNA and protein were shown to be present on NIE-115 cells, mediating apoptosis, perhaps via breakdown to adenosine [543], but more likely by direct activation [544]. P2U (i.e. $P2Y_2$ and/or $P2Y_4$) receptors, as well as $P1(A_2)$ receptors, have been identified on NCB-20 mouse neuroblastoma × Chinese hamster brain explant hybrid cells [545]. ATP was shown to inhibit atrial natriuretic peptide binding to R1-type receptors on human neuroblastoma NB-OK-1 cell membranes [546]. Neuropeptide Y was shown to modulate ATP-induced increase in $[Ca^{2+}]_i$ via the adenylate cyclase/PKA system in the CHP-234 cell line derived from a human neuroblastoma [547].

Extracellular ATP evoked two excitatory responses in hippocampal neuroblastoma cells (HN2): one opened receptor-operated, non-selective cation channels, perhaps

via P2X7 receptors, and the other caused a leftward (negative) shift in the Na^+ channel activation curve, probably via P2Y receptors [548]. The human SH-SY5Y neuroblastoma cell line expresses a functional P2X7 receptor that modulates voltage-dependent Ca^{2+} channel function [549]. Extracellular guanosine and guanosine triphosphates promote the expression of differentiation markers and induce S-phase cell cycle arrest in SH-SY5Y cells [550]. Agonist-induced stimulation of both A_1 and A_{2A} receptor induced neurite outgrowth and differentiation of SH-SY5Y neuroblastoma cells in vitro [551].

mRNAs for $P2Y_1$, $P2Y_4$ and $P2Y_6$ receptors were expressed in SK-N-BE(2)C human neuroblastoma cells, but Northern blot analysis revealed that $P2Y_6$ receptors were the predominant subtype [552]. In an abstract, the presence of functional $P2Y_1$ and P2X4 receptors (in addition to $P2Y_6$, $P2Y_{11}$, P2X5, P2X6 and P2X7 receptor protein) was claimed in human SK-N-MC neuroblastoma cells [553].

Neuroblastoma is the most common tumour in infancy and early childhood. Neuroblastoma and the sympathetic nervous system share a common embryological origin, the neural crest. C-1300 neuroblastoma arose spontaneously in mouse and resembles human neuroblastoma in many respects. Evidence has been presented that the sympathetic nervous system secretes a mitogenic trophic factor that enhances growth of C-1300 neuroblastoma cells in vivo [554]. It is now well established that ATP is released as a cotransmitter with NA in sympathetic nerves and that it often has powerful trophic actions (see [555]). Uridine induces differentiation of LAN-5 human neuroblastoma cells [556]. $P2Y_4$ receptors were claimed to participate in differentiation and cell death of human neuroblastoma SH-SY5Y cells [557]. Glucocorticoids inhibit P2X receptor-mediated Ca^{2+} influx via a PKA-dependent pathway in HT4 mouse neuroblastoma cells [558]. P2X7 receptors expressed by primary human neuroblastoma cells are uncoupled from their well-known cytotoxic effect, but rather support cell growth. This paradoxical effect seems to be due to an inability to induce caspase-3 activation [559]. The growth stimulation was partially due to the release of substance P from nucleotide-activated neuroblastoma cells. Adenosine is claimed to induce apoptosis in mouse neuroblastoma NIE-115 cells, but uptake of adenosine and its subsequent phosphorylation is required [560]. ATP can stimulate neurite outgrowth in mouse neuroblastoma neuro2a cells independent of other neurotrophic factors [561].

Mouse neuro-2a cells differentiated into neuronal-like cells after exposure to retinoic acid, which was associated with a decrease in expression of functional P2X7 receptors [562]. It was further shown that P2X7 receptor antagonists induced neurite outgrowth as did P2X7 receptor knock-outs and it was concluded that decreases in the expression of P2X7 receptors are associated with neuronal differentiation and that ATP release-activated P2X7 receptors are important in maintaining cell survival of N2a neuroblastoma cells. A study of neuro-2a cells from another laboratory [35] also showed that P2X7 receptor inhibition led to an increase in neurite formation and that P2X7 receptors are involved in the maintenance of neuroblastoma cells in the non-differentiated state. P2X7 receptors are expressed in human lingual nerve neuromas [563]. It has been suggested that there is a positive feedback mechanism mediated by P2X7 receptor-stimulated exocytotic release of ATP that would act on P2X7 receptors on the same or neighbouring cells to further stimulate its own release and negatively control mouse neuroblastoma Neuro2-a cells [564]. The therapeutic potential of P2X7 receptor antagonists for the treatment of neuroblastoma has been reviewed [565].

Benzodiazepines modulate adenosine A_2 receptor binding sites on 108CC15 neuroblastoma × glioma hybrid cells [566]. Prolonged exposure to A_2 receptor agonists was associated with a small, but significant degree of differentiation of IMR32 human neuroblastoma cells [567]. A_1 receptors have been identified in peritumoural zone around experimental F98 and C6 rat brain tumours [568].

In summary, $P2Y_2$, $P2Y_6$ and P2X7 receptors, which mediate cytotoxic effects, appear to be the dominant purinoceptor subtypes in most neuroblastoma cell lines.

Gliomas

Glioma is a general term for malignant tumours of glial cells and includes astrocytomas, oligodendrogliomas, medulloblastomas, Schwannomas, ependymonas and glioblastomas.

Adenosine triphosphatase was found to be localised on the cell membranes of gliomas over 35 years ago [569]. Later, an ecto-nucleotide pyrophosphatase (ectoNPPase) was identified for ATP metabolism by C6 glioma cells [570, 571] and ecto-5-nucleotidase [572]. Thyroid hormone upregulates ecto-5′-nucleotidase (CD73) in C6 cells [573]. CD73 has also been identified in human U138MG glioma cells [574]. CD73, a producer of extracellular adenosine, modulates U138MG glioma cell adhesion and tumour cell–extracellular matrix interactions [575]. Medulloblastoma is the most common malignant brain tumour in children and occurs mainly in the cerebellum. ATP was secreted from three malignant human cell lines and absence of CD73 in the D283 cell line, a metastatic medulloblastoma phenotype, suggested that high expression of CD73 could be correlated with a poor prognosis in patients with medulloblastomas [576]. Selective expression of NTPDase 2 modulates growth of rat C6 and COS-7 glioma cell lines in vivo [577]. Overexpression of NTPDase2 in C6 glioma cells promotes systemic inflammation and pulmonary injury [578]. Intracarotid, but not intravenous, administration of adenosine and ATP into intracerebrally transplanted RG-C6 tumours in rats selectively increased blood flow in the tumour, suggesting that they may be used to

enhance the delivery of anti-cancer agents to malignant brain tumours [579, 580].

Rat glioma C6 cells have been widely used for studies of gliomas. ATP was shown to stimulate phosphoinositide hydrolysis in C6 cells [581], suggesting that P2Y receptors were involved, as was supported by thapsigargin blockade of ATP-mediated increase in $[Ca^{2+}]_i$ [582]. It was suggested that the P2 receptor subtype in C6 cells was comparable to the P2T receptor of platelets, i.e. the $P2Y_{12}$ receptor [583]. UTP and ATP were equipotent in increasing $[Ca^{2+}]_i$ in C6 glioma cells, suggesting mediation via P2U (i.e. $P2Y_2$ and/or $P2Y_4$) receptors [584, 585]. There appeared to be two different signal transduction pathways for P2Y receptors in C6 cells, one is involved in inhibition of adenyl cyclase and the other in the induction of phosphoinositide turnover, indicating the involvement of two P2Y receptor subtypes [586, 587]. A later paper identified both $P2Y_1$ and $P2Y_2$ receptors involved in calcium signalling in C6 cells [588]. In addition to $P2Y_2$ receptors on C6 cells, ADP acts via $P2Y_1$ and $P2Y_{12}$ receptors, the former linked to PLC, while the latter is coupled to adenylate cyclase [589]. Cross-talk between $P2Y_1$ and $P2Y_{12}$ receptors has been implicated in growth and differentiation of C6 cells [590, 591]. A shift in receptor expression from $P2Y_1$ to $P2Y_{12}$ in long-term serum-deprived C6 cells appears to be a self-regulatory mechanism that promotes cell growth rather than differentiation and is a defense mechanism against the effects of serum deprivation [592]. Bradykinin increased resensitization of P2Y receptor signalling in glioma cells [593]. siRNA silencing of $P2Y_1$ receptors alters calcium signalling in C6 cells [594]. In recent papers, $P2Y_{14}$ receptor activity has been described in C6 cells [595].

Rat C6 glioma cells express functional P2X7 receptors [596] and ATP-induced cell death in mouse GL 261 glioma cells was claimed to be mediated by P2X7 receptors [597]. P2X7 receptor agonists produced cell death in the glioma radiosensitive cell line M059J, but the radioresistant glioma cell line, U138-MG, presented resistance to death when treated with either ATP or BzATP [598]. ATP released from glioma tumour cells may act as the regulator, via P2X7 receptor signalling, that increases macrophage inflammatory protein-1α and monocyte chemoattractant protein-1 expression in tumour-infiltrating microglia [599]. It was claimed that BzATP-mediated calcium signalling in C6 cells was mediated by P2Y (perhaps $P2Y_2$), rather than P2X7 receptors [36]. P2X4 receptors were identified in glioma tumour growth areas, but immunostaining showed that they were largely, if not entirely, localized on infiltrating macrophages and activated microglia [600].

The ATP-forming capacity at the surface of glioma cells was several times greater than that of normal cells [601] and evidence for ectopic aerobic ATP production in C6 glioma cell membranes has been presented recently [602]. Adenosine uptake and ATP release from C6 cells were demonstrated [603], probably via pannexin 1 channels in response to mechanical stress [604]. ATP stimulates chemokine production in C6 glioma cells via a store-operated calcium entry pathway, which was suggested to enhance tumour cell mobility and promote recruitment of microglia into developing tumours, thereby supporting tumour growth [605].

ATP induces c-fos expression in C6 cells by activation of P2Y receptors [606]. The $P2Y_1$ receptor agonist, 2-methythio ADP, markedly increased C6 glioma cell migration, while the selective $P2Y_1$ receptor antagonist, MRS2179, significantly inhibited migration [607]. The authors suggested that $P2Y_1$ receptor antagonists could be a novel therapeutic procedure to slow glioma progression. UTP and ATP, mediated by $P2Y_2$ receptors, elicited proliferation of C6 glioma cells via activation of the Ras/Raf/MEK/MAPK pathways [608] (see also [609]). Both adenosine and ATP were claimed to induce proliferation in human glioma cell lines U87MG, U251MG and U138MG [610]. cAMP-dependent differentiation of C6 glioma cells into astrocyte type II is characterised by inhibition of cell growth and induction of glial fibrillary acidic protein synthesis. Activation of the $P2Y_{12}$ receptor inhibited β-adrenergic receptor-induced differentiation and a $P2Y_{12}$ receptor antagonist abolished this effect [611]. ERK 1/2 activity was positively correlated with cell proliferation evoked by both $P2Y_1$ and $P2Y_{12}$ receptor agonists, but in serum-starved cells, the effect of ADP on ERK 1/2 was primarily mediated by $P2Y_{12}$ receptors [612]. The mechanisms underlying $P2Y_{12}$ receptor activation of C6 cells have been studied, and it was concluded that PKB activation proceeds through insulin growth factor I receptor cross-talk and requires activation of Src, Pyk2 and Rap1 [613]. A review discussing P2Y receptor-mediated proliferation and differentiation of glioma cells is available [614].

Growth inhibition has been reported for human WF glioma cells by 8-ClAdo [615] and for C6 glioma cells by N^6-substituted cAMP analogs [616]. mRNA and protein for A_1, A_2 and A_3 receptors were shown to be expressed by C6 cells [617]. It has been suggested that adenine nucleotides inhibit C6 cell growth via adenosine after breakdown by CD73 [618]. Hypoxia has been claimed to decrease adenosine A_1 receptors, but to increase A_{2A} receptors in C6 cells [619]. U138-MG human glioma cells and C6 rat glioma cells showed greater resistance to death induced by ATP when compared to normal hippocampal organotypic cell cultures, indicating that released ATP can induce cell death of the normal tissue surrounding the tumour, potentially opening space to the fast growth and invasion of the tumour [620]. On the other hand, extracellular ATP might also exert a trophic effect on glioblastoma growth, as shown by the observation that in vivo C6 glioblastoma growth is reduced by co-injection of apyrase [621].

Temozolomide (TMZ) is a DNA-damaging agent, which is widely used for treating primary and recurrent high-grade gliomas. It has been shown that TMZ induces an autophagy-associated ATP surge in U251 cells that protect them and may contribute to drug resistance [622]. Carnosine inhibits growth of cell isolates from human malignant glioma, and recently, carnosine has been shown to inhibit ATP production in both cells from freshly resected gliomas and from the T98G human glioma cell line [623].

Glioblastomas are mainly, but not exclusively, undifferentiated anaplastic cells. This is the most aggressive type of brain tumour derived from glial cells and is characterised by having a cancer stem cell subpopulation essential for tumour survival. It includes astrogliomas, which are undifferentiated cells, but also astrocytomas, which are differentiated cells. Their rapid enlargement destroys brain cells and raises intracranial pressure, causing headache, vomiting and drowsiness. ATP, acting via P2Y receptors, increased $[Ca^{2+}]_i$ in primary cultures of human glioblastoma cells [624]. It was claimed that ATP induces IL-1β release from T98G glioblastoma cells through a purinoceptor-independent mechanism [625]. Human U87 glioma cultures presented tumour spheres that express the markers of glioma cancer stem cells. Extracellular ATP reduced tumour sphere growth and cancer stem cell population in the glioblastoma cells [626].

An A_2 receptor agonist increased the release of IL-8, an angiogenic factor, from the glioblastoma cell line U87MG, and while mRNA transcripts for A_1, A_{2A} and A_{2B} were identified in these cells, only A_{2B} receptors appeared to be functional; further, hypoxia increased A_{2B} receptor mRNA and A_{2B} antagonists inhibited tumour angiogenesis [627]. Adenosine attenuates growth of mouse glioblastoma G1361 cells acting via A_1 receptors on microglia [628]. Adenosine modulates VEGF expression via hypoxia-inducible factor-1 in human hypoxic U87MG and A172 glioblastoma cells via A_3 receptors [629]. Modulation of metalloproteinase-9 in U87MG cells via A_3 receptors has been reported [630]. Pulsed electromagnetic field exposure significantly increased the anti-tumour effect mediated by A_3 receptors in a human glioblastoma cell line [631]. Data have been presented to suggest that A_3 receptor agonists may be potential therapeutic agents for the induction of apoptosis in human glioma cells [632].

ATP, after breakdown to adenosine, increases intracellular cAMP in human 1321NI astrocytoma cells [633, 634]. However, later papers showed that high concentrations of ATP, acting via P2 receptors, stimulate proliferation of SKMG-1 and U373 human astrocytoma cells [635] or inhibition of proliferation of 132NI astrocytoma cell [636]. $P2Y_1$ receptors mediate stimulation of MAPKs and induction of apoptosis in 132NI astrocytoma cells [637]. The P2X7 receptor agonist, BzATP, induced ERK 1/2 phosphorylation in human astrocytoma cells over-expressing the recombinant rat P2X7 receptor [638]. $P2Y_6$ receptors mediate activation of PKC to protect 132N1 astrocytoma cells against tumour necrosis factor-induced apoptosis [639]. $P2Y_{12}$ receptors were shown to be expressed in 132N1 cells mediating Ca^{2+} signals, which may be crucial for regulating cell proliferation and differentiation [640]. An enhanced green fluorescent protein-tagged human $P2Y_2$ receptor was expressed in 1321N1 astrocytoma cells [641]. $P2Y_{14}$ receptors were also identified on 1321N1 cells, leading to the release of UDP-glucose [642]. Extracellular osmolarity modulates G protein-coupled receptor-dependent ATP release from 1321N1 astrocytoma cells [643].

A_{2B} receptors mediate an increase in IL-6 mRNA and protein synthesis in the human astrocytoma cell line U373MG [644]. An A_3 receptor mediates cell spreading and reorganisation of the actin cytoskeleton in human ADF astrocytoma cells [645, 646]. A_3 receptor agonists mediated desensitization, internalization and down-regulation of the A_3 receptors in human astrocytoma ADF cells [647]. Extracellular adenosine, acting via A_1 receptors, activates caspase-9 and then caspase-3 via two independent pathways, leading to cell death of RCR-1 rat astrocytoma cells predominately by apoptosis [648]. ADA inhibition induces apoptosis in a human astrocytoma cell line [649].

Phaeochromocytoma

Phaeochromocytoma describes a tumour of adrenal medulla (or sympathetic nervous system), characterised by an excess of NA and hypertension. PC12 cells are a clonal line of rat phaeochromocytoma and have been extensively studied. They secrete NA, dopamine (DA) and acetylcholine (ACh) by a Ca^{2+}-dependent process.

Extracellular ATP stimulated NA secretion from PC12 cells [650, 651], but also uptake of NA [652, 653]. ATP-activated inward current in PC12 cells was demonstrated with an agonist potency order of ATP > ATPγS > ADP, while adenosine and α,β-methylene ATP were inactive [654]. Suramin antagonised the ATP-activated current [655] and catecholamine secretion [656]. ATP stimulated the release of DA from PC12 cells [657, 658], which was suppressed by reactive blue 2 [657]. ATP and nicotine both activate an inward current, but the binding sites and the open states of the channels appear to be different [659].

The next step was to try to identify the purinoceptor subtype(s) located on PC12 cells (see, for example, [651, 660–663]). It was proposed that PC12 cells express at least two P2Y receptor subtypes: a P2Y subtype that leads to depletion of intracellular Ca^{2+} and NA release and a P2U (i.e. $P2Y_2$ and/or $P2Y_4$) receptor [664, 665]. The presence of P2X receptors on PC12 cells was first suggested in 1996 [666, 667]. Imipramine, a tricyclic antidepressant, inhibited,

via P2X2 receptors, the ATP-evoked increase in $[Ca^{2+}]_i$ and DA release by PC12 cells [668]. In a later paper, fluoxetine, another antidepressant, was also shown to inhibit ATP-induced increase in $[Ca^{2+}]_i$ in PC12 cells [669]. Data have been presented to suggest that in undifferentiated PC12 cells, ATP acts via P2X4 receptors, but after nerve growth factor (NGF) treatment, the differentiated cells respond largely via $P2Y_2$ receptors [670]. Both P2X2 and P2X4 receptor mRNA was shown to be present on PC12 cells and ATP, acting via both P2X and P2Y receptors, elevated $[Ca^{2+}]_i$, thereby facilitating catecholamine secretion [671]. Further, they showed that Na^+ entry through P2X2 receptors effectively activated L-type voltage-sensitive Ca^{2+} channels. Transcriptional regulation of P2X2 receptors on PC12 cells by retinoic acids has been reported [672]. Dehydroepiandrosterone sulphate, the major circulating steroid in humans, suppresses P2X, but not P2Y, receptor-coupled responses of PC12 cells [673]. ATP triggers catecholamine release from PC12 cells via P2 receptors that desensitize; thus, habituation is increased by UTP [674]. It has been suggested that ATP-induced increase in $[Ca^{2+}]_i$ is mainly due to the release of mitochondrial Ca^{2+} through Na^+–Ca^{2+} exchangers in PC12 cells [675]. The membrane localization of PKCα is regulated by Ca^{2+} influx through P2X channels and phosphatidylinositol 4,5′-biphosphate in NGF-differentiated PC12 cells [676].

An RT-PCR and electrophysiological study of P2X receptors in PC12 cells showed that only functional P2X2 receptors were expressed in undifferentiated cells, but all seven P2X receptor subtypes were expressed in NGF-differentiated cells [677]. Another paper was consistent with these findings showing that NGF-stimulated differentiation of PC12 cells induced changes in P2 receptor expression and nucleotide-stimulated catecholamine release [678]. In particular, P2X receptor-selective agonists caused greater NA release from differentiated compared to undifferentiated cells, and receptor protein expression was increased for P2X1-4 receptors, but not P2Y receptors. P2Y receptors on PC12 cells mediate the actions of ATP and UTP to activate MAPK activity and promote the tyrosine phosphorylation of RAFTK, the epidermal growth factor receptor [679]. High concentrations of free fatty acids increased the expression of P2X7 receptors in PC12 cells via activation of the p38 MAPK signalling pathway, enhancing the release of IL-6 [680].

PC12 cells were claimed to express $P2Y_1$-like receptors that mediate inhibition of voltage-activated Ca^{2+} currents in PC12 cells [681]. It has been reported that processes of differentiated PC12 cells possess $P2Y_{12}$ receptors mediating inhibition of stimulation-evoked calcium entry [682]. Attenuation of P2Y receptor-mediated control of Ca^{2+} channels in PC12 cells by anti-thrombotic drugs has been claimed [683]. ADP-activated $P2Y_1$ and $P2Y_{12}$ receptors on PC12 cells are activated by spontaneous release of nucleotides, while ATP/UTP-sensitive $P2Y_2$ receptors require an excess of depolarisation-evoked release to become activated [684]. A later paper showed that spontaneous release of nucleotides may occur independently of vesicle exocytosis, whereas depolarization-evoked release of ATP relies predominantly on exocytotic mechanisms [685]. It has been suggested that regulation of differentiation and cell survival of PC12 cells is mediated by the P2Y-like G protein-coupled GPR17 receptor [686]. ATP enhanced differentiation of PC12 cells by activating PKCα interactions with cytoskeletal proteins [687]. Sustained elevation of $[Ca^{2+}]_i$ via P2X receptors causes changes in gene expression via activation of the transcription factor nuclear factor of activated T cells in PC12 cells [688].

Adenosine appears to be an endogenous regulator of tyrosine 3-monooxygenase activity in cell suspensions prepared from transplantable rat phaeochromocytoma [689], but this is defective in adenosine kinase-deficient PC12 cells [690]. Adenosine, acting through P1 receptors coupled to stimulation of adenylate cyclase, enhances the release of NA and ACh from PC12 cells [691]. 2-ClAdo increases the specific activity of choline acetyltransferase in PC12 cells [692]. Later, an A_{2A} receptor was identified on PC12 cells [693–695], which inhibited ATP-induced Ca^{2+} influx [696]. Chronic hypoxia reduced A_{2A} receptor-mediated inhibition of calcium currents in PC12 cells [697] and induced neurite outgrowth [698]. Adenosine increased DA metabolism in PC12 cells, which may have implications in relation to dopaminergic deficit in Parkinson's disease [699]. A_1 receptor activation was reported to inhibit neurite formation in PC12 cells [700]. Induction of neurite outgrowth in PC12 cells by the bacterial nucleoside, N6 methyldeoxyadenosine, was mediated by A_{2A} receptors [701]. A_{2A} receptor ligands and proinflammatory cytokines induce PC12 cell death through apoptosis [702]. Adenosine is an active component of *Antrodia cinnamomea*, a medicinal fungus in Taiwan, which prevents PC12 cells from serum deprivation-induced apoptosis through the activation of adenosine A_{2A} receptors [703]. AMP N_1-oxide, a unique compound of royal jelly, induces neurite outgrowth of PC12 cells via A_{2A} receptors [704]. Adenosine potentiates ATP-evoked DA release from PC12 cells [705].

Facilitation of the ATP-activated current in PC12 cells by 5-hydroxytryptamine and DA has been reported [667, 706] and enhancement of ATP-evoked DA release by zinc [707] and cadmium [708]. Reduction of ACh-activated current by ATP has also been observed [709]. ATP, acting through P2Y receptors, leads to the release of arachidonic acid from PC12 cells [710]. Diadenosine tetraphosphate, probably acting via a P2Y receptor, increased $[Ca^{2+}]_i$ in PC12 cells [711].

Both catecholamines and ATP were released from PC12 cells in response to elevated intracellular concentrations of calcium [712]. There is enhancement of ATP levels in PC12 cells by the actions of extracellular adenosine [713]. Autoinhibition of

transmitter release from PC12 cells (and sympathetic neurons) through $P2Y_{12}$ receptor-mediated inhibition of voltage-gated Ca^{2+} channels has been reported [714]. Small transient inward currents were caused by quantal release of endogenous ATP by depolarised PC12 cells in close juxtaposition to the recorded cells [715]. There was enhancement of cellular ATP levels in PC12 cells by 2,5-dideoxyadenosine, a P-site inhibitor of adenylate cyclase [716]. Endothelin-1 inhibited the release of ATP from PC12 cells via ET_B receptors by attenuation of the influx of extracellular Ca^{2+} through L-type channels [717]. β-Nicotinamide adenine dinucleotide was released together with ATP and DA from PC12 cells, but probably with different sites of vesicular release [718].

CD73 activity was inhibited in PC12 cells and was stimulated by treatment with NGF [719]. It was reported that CD73 played a crucial role in differentiation and survival of PC12 cells [720]. Extracellular ATP enhanced lipid peroxidation in PC12 cells and it was suggested that ATP may contribute to cell death by an oxidative mechanism involving lipid peroxidation [721]. Guanosine triphosphate and guanosine synergistically enhance NGF-induced neurite outgrowth from PC12 cells [722–724]. A later paper showed that guanosine stimulated neurite outgrowth in PC12 cells via activation of heme oxygenase and cyclic guanosine monophosphate [725]. ATP activates transcription factor AP1, a regulatory protein that converts extracellular signals into changes in gene expression programs and may modulate expression of target genes involved in cell death pathways in PC12 cells [726]. ATP inhibited starvation-induced apoptosis via P2X2 receptors in differentiated PC12 cells [727]. L-type Ca^{2+} channels and P2X2 receptor cation channels participated in calcium-tyrosine kinase-mediated PC12 growth cone arrest [728]. Uridine enhances neurite outgrowth of NGF-differentiated PC12 cells, perhaps through UTP as an agonist at $P2Y_2$ receptors [729]. Neurite outgrowth in PC12 cells is also enhanced by ATP released into the medium through connexin hemichannels [730].

PC12 cells develop normal characteristics of sympathetic neurons after treatment with NGF and P2 receptor antagonists prevent NGF-dependent neuritogenesis [731]. P2 receptor agonists can behave as neurotrophic factors and interact with NGF signalling in neurite outgrowth and survival of PC12 cells [732, 733]. ATP-induced mitogenesis is inhibited by PLD2 in PC12 cells [734]. 2-ChloroATP exerts anti-tumoural actions (cell cycle arrest or cell death) in PC12 cells, although it was claimed that this was not mediated by P2 receptors [735]. Ca^{2+} influx through P2X receptors induces actin cytoskeleton reorganisation by the formation of cofilin rods in neurites of PC12 cells [736].

The parkin gene is one of the eight genes responsible for Parkinson's disease. Parkin has been shown to potentiate ATP-induced currents via activation of P2X receptors in PC12 cells, suggesting that parkin may play a role in synaptic activity [737].

Phthalates are environmental pollutants and buylbenzylphthalate blocks purinoceptor-mediated Ca^{2+} signalling in PC12 cells [738]. Toluene disocynate, another toxic pollutant, suppresses calcium signalling produced via P2X receptors in PC12 cells [739].

Cancer pain

There are an increasing number of reports implicating the involvement of purinergic signalling in cancer pain. It was suggested that the exceptionally high levels of ATP contained in tumour cells may be released by mechanical rupture to activate P2X3 receptors on nearby sensory nerve fibres [350]. P2X3 receptor antagonists are one of the targets being explored against cancer pain [740]. Increased expression of P2X3 receptors on calcitonin gene-related peptide (CGRP)-immunoreactive epidermal sensory nerve fibres in a bone cancer pain model was reported [354] and in other tumours that are responsive to mechanical stress. In bone tumours, the mechanical strength of the bone is reduced and antagonists that block ATP receptors in the richly innervated periosteum might reduce movement-associated pain. The hyperalgesia associated with tumours appears to be linked to increase in expression of P2X3 receptors in nociceptive sensory neurones expressing CGRP by analogy with that described for increased P2X3 receptor expression in a model of inflammatory colitis. Increased expression of P2X3 receptors was also shown to be associated with thermal and mechanical hyperalgesia in a rat model of squamous cell carcinoma of the lower gingival [741]. Responses mediated by both P2X3 and P2X2/3 receptors on sensory neurones are inhibited by μ-opioid receptor agonists showing that P2X and μ-opioid receptors are functionally coupled on sensory neurones [742]. In a later paper, it was shown that P2X3 receptors on sensory neurones co-cultured with cancer cells exhibit a decrease in opioid sensitivity [341].

Radiotherapy is effective in relieving bone pain and it has been claimed from studies of a hind paw model of cancer pain by transplanting a murine hepatocarcinoma into the periosteal membrane of the foot that P2X6 receptor expression in the spinal cord was increased fivefold in the tumours, but that this was reversed following radiation [356].

Orthotopic inoculation of B16-BL6 melanoma cells into the hind paw of mice produced spontaneous licking of the tumour-bearing paw, an indication of pain [743]. P2X3 receptor antagonists suppressed the spontaneous licking and it was concluded that P2X3 receptors were involved in skin cancer pain, due to the increased release of ATP and increased expression of P2X3 receptors in the sensory neurons. In an elegant study, systemic blockade of P2X3 and P2X2/3

receptors was shown to attenuate bone cancer pain behaviour in rats [744], and in a later paper, the P2X3 and P2X2/3 antagonist, A317491, was shown to transiently attenuate cancer-induced bone pain in mice [745]. A recent review that discusses the role of purinergic receptors in cancer-induced bone pain is available [746]. μ- and δ-Opioid receptors are expressed on isolectin (IB) 4^- (that expresses some P2X3 receptors) and $IB4^+$ (that expresses most P2X3 receptors) neurons, respectively, which control thermal and mechanical pain, and it was shown that $IB4^+$ and $IB4^-$ neurones were differentially involved in oral squamous cell carcinoma-related pain [747]. Using δ-opioid agonists and P2X3 receptor antagonists, it was shown that $IB4^+$ neurones play a key role in cancer-induced mechanical allodynia, but not in thermal allodynia.

It has been shown that P2X7 receptor knock-out mice were susceptible to bone cancer pain and had an earlier onset of pain-related behaviours compared with cancer-bearing, wild-type mice [748]. They showed further that the P2X7 receptor antagonist, A-438079, failed to alleviate the pain-related behaviours and concluded that P2X7 receptors play a negligible role in bone cancer pain. Evidence has been presented that $P2Y_1$ receptors in the spinal cord and DRG may mediate bone cancer pain through the ERK pathway [749].

Concluding comments

Overwhelming clinical evidence supports the notion that the approach to a cancer cure must be based on targeting multiple receptors and pathways, and that the best results are obtained when physiological mechanisms for cancer cell elimination are seconded (as in the case of immunochemotherapy) rather than being ignored. Purinergic signalling is a ubiquitous, crucial, pathway responsible for cell-to-cell communication in physiology and more so in pathology. Recent developments in the construction of reliable molecular probes for the measurement of extracellular ATP have unequivocally demonstrated that ATP at sites of inflammation or neoplasia can reach hundreds of micromolar concentrations [65, 66, 750]. The different P1 and P2 receptor subtypes expressed to a different extent by different cells and the large change in concentration that adenosine and ATP may undergo in physiological and pathological conditions offers an enormous plasticity to purinergic signalling. We are now starting to harvest the therapeutic potential of this system, but it is clear that only a deep knowledge of the molecular and biochemical details of involved pathways will allow us to exploit it in full to our benefit. In particular, it will be crucial to identify those conditions where trophic effects on tumour cells due to extracellular ATP overweight the well-known cytotoxic activity due to activation of specific P2 receptors such as P2X7. This is a crucial issue since several in vitro data and scattered in vivo evidence show that ATP may stimulate tumour cell growth [50, 751]; in addition, some tumours seem to be refractory to killing via the P2X7 receptor [559]. It will be necessary to thoroughly investigate in pre-clinical settings the effect of the administration of ATP (or P2X7-selective agonists or antagonists) via routes that better mimic the current procedures of anti-cancer drug administration in humans, e.g. intravenous infusion (see [14, 751–753]).

The picture is made even more complex by the growing awareness that P2 receptor activation/inhibition has a profound immunomodulatory function and thus shapes in a crucial fashion the type and number of tumour-infiltrating inflammatory cells (see [17, 63, 82, 83, 85, 754–758]). Therefore, it will be necessary to monitor the possible adverse effects caused by the systemic administration of P2-targeted drugs. Nevertheless, as our understanding of purinergic signalling increases, so does the range of malignancies found to be dependent on this messenger system. 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, although significant differences between tumour types must be recognised. Much of the evidence has so far been derived from in vitro studies, with less information from in vivo animal experiments and little from human observational studies and randomized, controlled trials; thus, the need for more such studies to be performed is great, since it is not possible, based on existing in vitro and in vivo studies, to predict clinical effects. Nevertheless, studies have shown functional roles for the P2X5 and/or $P2Y_{11}$ receptors, while a contributory effect of P2X7 receptors cannot be discounted. Selective targeting of these aberrant pathways would allow for the development of novel therapeutic agents that could not only treat the primary malignancy, but also improve the systemic symptoms associated with advanced malignancy. Both irradiation and chemotherapy appear to induce the release of ATP from tumour cells, which could exert cytotoxic effects by causing cell death via P2X7 receptors. Recent studies have shown that high levels of ATP are released from tumour cells that activate inflammasomes, thereby triggering a pro-inflammatory cascade leading to the activation of immune responses. ATP secretion from tumour cells is claimed to be involved in immunogenic cancer cell death [759].

One of the most important immunosuppressive regulatory pathways is the phosphohydrolysis of extracellular ATP to adenosine by the high levels of ectonucleotidase expressed by tumour cells (e.g. CD73), a possible novel target for cancer therapy. CD73 is a potent suppressor of anti-tumour immune responses [760]. Blockade of A_{2A} receptors has been proposed as a target for tumour immunotherapy that synergizes with other immunomodulatory approaches currently in

clinical trials [761]. A_{2A} receptor signalling is required for T cell homeostasis and control of tumour growth [762]. A_{2B} receptor signalling in antigen-presenting cells suppressed anti-tumour adaptive immune responses [763]. CD73-deficient mice have increased anti-tumour immunity and are resistant to experimental metastasis [764] (see also [754, 765]).

The serine-threonine kinase, Akt, plays a central role in propagating growth signals, metabolism and cell survival, making it a potential therapeutic target for cancer [766]. It was shown in this paper that ATP competitive inhibitors induced increased phosphorylation of Akt, suggesting a mechanism for regulating kinase activity through nucleotide binding. Since ATP is a naturally occurring small molecule, its radiolabelled form, [^{32}P]ATP, poses advantages as a potential anti-cancer therapeutic agent and it was shown to inhibit the growth of xenografted tumours in nude mice [767]. Collectively, these evidences highlight the crucial role of purinergic signalling in cancer growth and dissemination and underline the, as yet, largely unexploited therapeutic potential of P2 receptor targeting. A_3 receptors have been proposed as a therapeutic approach in cancer [768].

Hematopoietic stem cell transplantation is being developed as a therapeutic option for patients with hematologic malignancies; release of ATP from CD4 cells in whole blood was increased, which contributed to the clinical management of patients with hematologic malignancies [769]. A single cell, enhanced fluorescence ATP biosensor was developed recently to monitor ATP release from heterogeneous cancer populations in real time [770].

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