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# P2Y purinergic receptors regulate the growth of human melanomas

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**Abstract**

Adenosine 5′-triphosphate is known to function as a potent extracellular messenger producing its effects via a distinct family of cell surface receptors. Different receptor subtypes have been shown to modulate different cellular functions such as proliferation, differentiation and apoptosis. We investigated the functional expression and proliferative action of metabotropic P2Y receptors in human melanoma tissue and cells. Expression of functional $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptor subtypes was established by reverse transcriptase polymerase chain reaction, immunohistochemistry and intracellular calcium measurements using a Fluorometric Imaging Plate Reader. Incubation of A375 melanoma cells with the $P2Y_1$ receptor-selective agonist 2-methylthioadenosine-5-diphosphate caused a decrease in cell number which was dose-dependent, whereas incubation with the $P2Y_2$ receptor agonist uridine triphosphate caused a dose-dependent increase in cell number. The action of extracellular nucleotides on P2Y receptors was shown to mediate the growth of melanomas and the $P2Y_1$ receptor is a putative target for melanoma therapy.


*Keywords:* Cancer; Melanoma; P2Y receptor; ATP

## 1. Introduction

Melanoma is an important cutaneous malignancy as it accounts for 95% of all deaths from skin disease [1]. The incidence of melanoma is increasing [2] and the outcome for patients with advanced disease remains poor as there is resistance to current methods of treatment [3], therefore new treatment strategies are needed.

There is increasing evidence that purinergic signalling can have prolonged effects on cell growth and proliferation [4,5]. The current classification of purinergic receptors, suggested by Burnstock [6], divides purinergic receptors into two groups. P1 receptors are selective for adenosine [7] while P2 receptors are selective for adenosine 5′-triphosphate (ATP) [8], P2 receptors are further subclassified by their pharmacological properties and transduction mechanisms into P2X and P2Y receptors [9,10].

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P2X receptors are ligand-gated ion channels which are activated by extracellular ATP to elicit a flow of cations [11]. Seven subtypes of these ionotropic receptors have been identified ($P2X_1$–$P2X_7$). P2X receptors are mainly expressed in the nervous system, platelets and in smooth muscle cells. P2Y receptors, however, belong to the family of G-protein-coupled receptors [12] and the principal signal transduction pathway involves phospholipase C, which leads to the formation of inositol 1,4,5-triphosphate ($IP_3$) and mobilization of intracellular calcium [13]. Depletion of intracellular calcium stores leads to calcium-mediated calcium influx across the cell membrane [14]. Metabotropic P2Y receptors have been described in a wide range of normal tissues including blood vessels [4], bone [15], the gastrointestinal tract [16] and the epidermis [5].

The anti-cancer activity of adenine nucleotides was first described by Rapaport [5,17]. Intraperitoneal injection of ATP into tumour bearing mice resulted in significant anti-cancer activity against several fast growing, aggressive carcinomas [18]. The presence of P2Y receptors has been described in several cancer types including oesophageal [19], prostate [20], lung [21], sarcoma and colorectal cancer cell lines [22]. Both P1 [23,24] and $P2X_7$ receptors [25] have previously been described in melanoma. P2Y receptors have not been examined in melanomas to date. Therefore we investigated the role of P2Y receptor-mediated effects of extracellular nucleotides in human melanoma tissue and cells.

## 2. Materials and methods

### 2.1. Materials

The melanoma cell line A375 [26] was obtained from the Wellcome Trust Functional Genomics Cell Bank (St Georges Hospital Medical School, London, UK). Cell culture medium and reagents were purchased from Sigma (Poole, UK). SV Total RNA Isolation system was purchased from Promega (WI, USA) and Ready-To-Go RT-PCR beads were purchased from Amersham Biosciences (NJ, USA). RT-PCR primers were obtained from Invitrogen (Paisley, UK).

### 2.2. Cell culture

Melanoma cells were grown in 90% Dulbecco's Modified Eagle's Medium (DMEM) and 10% heat inactivated fetal calf serum supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) in 75 $cm^2$ tissue culture flasks (Corning, NY, USA). Cells were incubated at 37 °C in 5% $CO_2$/95% air and were subcultured at 70% confluence. Cell viability was determined using the trypan blue exclusion method.

### 2.3. Immunohistochemistry of paraffin embedded specimens of melanoma

A total of 14 specimens of melanoma were examined for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors. Paraffin blocks were sectioned at 4 μm on a Reichert-Jung Microtome, and sections were taken on Snowcoat Extra slides (Surgipath, Cambridgeshire, UK), then dried in an oven for 2 h at 60 °C. Sections were dewaxed and rehydrated using xylene and graded concentrations of ethanol. Antigen retrieval was performed by microwaving for 10 min in a solution of 1 mM ethylenediamine tetraacetic acid (Tris–EDTA) at pH 9.0. Endogenous alkaline phosphatase was blocked by 20 min of incubation in 20% acetic acid. Sections were washed and then incubated with avidin D blocking solution, biotin blocking solution and 1:5 normal swine serum (Vector Laboratories).

Polyclonal anti-$P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor antibodies, corresponding to a 14–16 peptide sequence of the intracellular portion of the transmembrane receptors, were obtained from Alomone Laboratories (Jerusalem, Israel). They were kept frozen at a stock concentration of 0.6 mg/ml and used at a dilution of 1:100. 100 μl of anti-P2Y receptor antibody, diluted 1:100, was applied for 12 h at 4 °C. One hundred microlitres of biotinylated anti-rabbit antibody (DAKO E0353), diluted 1:200 in DAKO ChemMate was applied for 30 min followed by 100 μl of streptavidin alkaline phosphatase (Vector SA5100) diluted 1:200 in DAKO ChemMate for 30 min. Vector Red substrate (Vector Alkaline phosphatase substrate, SK5100) made up in 200 mM Tris–HCl (pH 8.2) was then applied for 10 min. Positive staining appeared bright pink, nuclei were counterstained with hematoxylin (purple). All sections were subsequently

dehydrated, cleared and mounted. Negative controls were performed by either omission of the primary antibody or preabsorption of the primary antibody with the corresponding peptide sequence.

### 2.4. Immunocytochemistry

A375 cells were grown in culture on chamber slides (Nunc, IL, USA) until 50% confluent. They were fixed in 4% formaldehyde in 0.1 M phosphate buffer for 2 min. Non-specific binding sites were blocked by a 20 min pre-incubation with 10% normal horse serum (NHS) in 0.1 M phosphate buffer containing 0.05% merthiolate, followed by incubation with primary P2Y receptor antibody with 0.2% Triton, for 12 h at 4 °C. Subsequently, the slides were incubated with donkey anti-rabbit Cy3 (Jackson Immunoresearch, PA, USA) diluted 1:300 with 1% NHS in phosphate buffer. Slides were then mounted with Eukitt (BDH laboratories, Dorset, UK) and examined. Control experiments were carried out with the primary antibody being omitted from the staining procedure or the primary antibody preabsorbed with the corresponding peptide. All other reagents were obtained from Sigma (Poole, UK).

### 2.5. Reverse transcriptase polymerase chain reaction

The expression of mRNA encoding the P2Y receptors in A375 melanoma cells was determined using reverse transcriptase (RT) and the polymerase chain reaction (PCR). Total RNA was isolated from A375 cells grown in culture using the SV Total RNA Isolation System. The RT-PCR reactions were performed using Amersham Biosciences Ready-To-Go RT-PCR beads. Reverse transcriptase was carried out on 1 μg of RNA for 30 min at 42 °C. The polymerase chain reaction cDNA samples were initially denatured for 5 min at 95 °C prior to initiating the PCR step. The P2Y primer sequences used were as follows: $P2Y_1$ 5′-cgg tcc ggg ttc gtc c-3′ and 5′-cgg acc ccg gta cct-3′ product size 527 base pairs; $P2Y_2$ 5′-ctc tac ttt gtc acc acc agc g-3′ and 5′-ttc tgc tcc tac agc cga atg tcc-3′ product size 637 base pairs; $P2Y_4$ 5′-cca cct ggc att gtc aga cac c-3′ and 5′ gag tga cca ggc agg gca cgc-3′ product size 424 base pairs and $P2Y_6$ 5′-cgc ttc ctc ttc tat gcc aac c -3′ and 5′-cca tcc tgg cgg cac agg cgg c -3′ product size 364 base pairs as previously described [27]. The amplification reaction was conducted under the following conditions: 95 °C for 30 s, the optimal annealing temperature for 30 s (54 °C for $P2Y_1$ and $P2Y_4$; 62 °C for the $P2Y_2$ and $P2Y_6$) and 72 °C for 1 min, run for 35 cycles. Amplification products were separated by electrophoresis and visualised by ethidium bromide staining. The presence of possible contaminants was investigated in all using control RT-PCR reactions in which, either mRNA had been omitted, or heating to 95 °C had inactivated the reverse transcriptase. Each reaction was repeated at least three times each from a separate mRNA preparation.

### 2.6. Measurement of intracellular calcium changes

A375 human melanoma cells were grown in 96-well plates (BD Falcon, NJ, USA) to confluence. Cells were loaded with the fluorescent dye Fluo-4 AM (Molecular Probes, Leiden, The Netherlands) at a concentration of 2 μM for 30 min at room temperature in a bath solution containing: 20 mM HEPES, 140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $K_2HPO_4$, 1 mM $CaCl_2$ and 10 mM D-Glucose with the pH adjusted to 7.4 with 1 M NaOH. For the experiments with calcium-free buffer, the $CaCl_2$ was omitted and 0.1 mM EGTA was added. To remove excess dye that had not been taken up by the cells, plates were washed three times with bath solution. The plates were placed into a Fluorometric Imaging Plate Reader (FLIPR-Molecular Devices, CA, USA) where they were incubated at 37 °C, prior to pre-incubation with the purinergic anatagonists pyridoxalphosphate-6-azophenyl-2′,4′-disulfonicacid (PPADS) and $N^6$-methyl-2′deoxyadenosine 3′,5′-bisphosphate (MRS 2179) and the addition of the P2Y agonists ATP, uridine triphospahte (UTP), uridine diphospahte (UDP) and 2-methylthioadenosine-5-diphosphate (2MeSADP). The cells and dye were excited by an argon-ion laser source at a wavelength of 488 nm and emission was measured at 520 nm at 1 s intervals. The change in fluorescence ($F$) measured was plotted as a change from average fluorescence ($F_0$) prior to the addition of agonist.

### 2.7. Cell proliferation assay

Cells were seeded onto a 96 well plate at a density of 250 cells/well (2500 cells/ml with 100 μl of cell

suspension per well). This seeding density gave the best growth curve over a 96 h period, 24 h after seeding the medium was aspirated and fresh medium containing either P2Y receptor agonist or just medium (control) was added to the well plates. ATP, 2-MeSADP, UTP, UDP were added in concentrations from 1 to 100 μM. The pH of the drug solutions prepared in the culture medium was adjusted to 7.36–7.44 prior to addition to the cells.

Changes in cell number were quantified by a colourimetric assay using crystal violet [28] and read using a spectrophotometric plate reader (Labtech, East Sussex, UK) at 24, 48 and 72 h after addition of purinergic agents.

For the colourimetric assay, a solution of 0.5 g crystal violet, 0.85 g NaCl, 5 ml 10% formal saline, 50 ml absolute ethanol, 45 ml distilled water was used. Medium was aspirated from the wells and 100 μl of the colourimetric assay mixture was added to each well and incubated at room temperature for 10 min. This mixture allowed simultaneous fixation of cells and penetration of the crystal violet dye into the cells. After washing three times with phosphate-buffered saline (PBS), 33% acetic acid was used to elute colour from the cells and optical density was read at 570 nm using the spectrophotometric plate reader. To confirm that the optical density of the wells correlated with cell number, a control assay was performed for each experiment where known numbers of cells were seeded in ascending seeding densities and the plate read as soon as the cells had attached. Cell number versus optical density was plotted. The $R^2$ value of the trend line was always greater than 0.98.

### 2.8. Photography

The results were analysed using a Zeiss Axioplan high definition light microscope (Oberkochen, Germany) mounted with a Leica DC 200 digital camera (Heerbrugg, Switzerland).

### 2.9. Statistical analysis

Each experiment was repeated at least three times each with three to eight samples. Data analysis was performed using Microsoft Excel XP Professional and GraphPad Prism 3.0 software. Comparisons of multiple means was performed by analysis of variance (ANOVA), the difference between samples was considered as significant when $P$-value was $<0.05$.

## 3. Results

### 3.1. P2Y receptors are expressed in tissue sections of melanoma

Positive labelling for P2Y receptor expression was present in all specimens of melanoma examined. The staining was uniform throughout with all melanoma cells appearing to express $P2Y_1$ and $P2Y_6$ receptors (Fig. 1A and D). $P2Y_2$ receptor staining was present, but was localised at the proliferating margins of the melanoma (Fig. 1B). No positive staining for $P2Y_4$ receptor protein was present in any of the specimens. As an internal positive control, $P2Y_1$ and $P2Y_2$ receptors were also appropriately expressed in the basal layer of the epidermis (Fig. 1A and C) as previously described [5].

### 3.2. P2Y receptors are expressed in A375 human melanoma cells grown in culture

There was cell membrane expression of $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors by A375 melanoma cells grown in culture; no $P2Y_4$ receptors were seen (Fig. 2). In addition there was some punctuate immunoreactivity present inside the cells, this is the visualisation of endocytosis of P2Y receptors. Internalization of metabatropic P2Y receptors is a recognized and well described feature of these receptors [29]. Both pre-absorption with the corresponding peptide and omission of the primary antibody were performed as controls which resulted in minimal immunoreaction.

### 3.3. mRNA for P2Y receptors is present in melanoma cells

RT-PCR analysis of the total RNA extracted from the A375 melanoma cells showed a positive band at the expected sizes for all four P2Y receptors examined (Fig. 3). $P2Y_4$ mRNA was detected; however, since there was no specific immunostaining for $P2Y_4$ receptors in either cells or tissue, it is unlikely

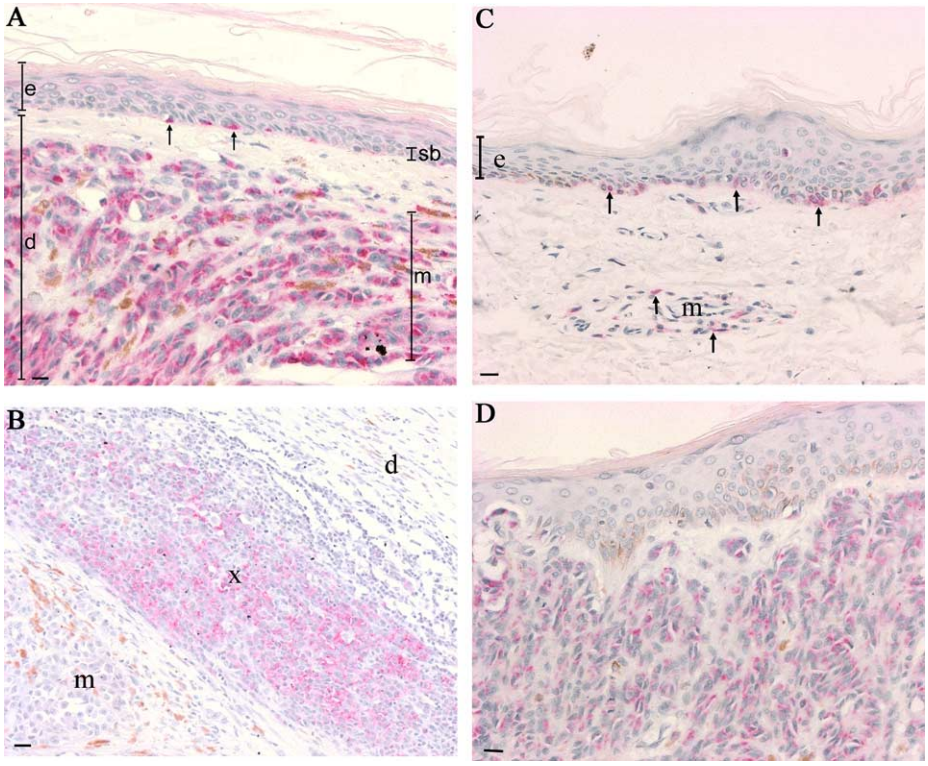


Fig. 1. P2Y receptors are expressed in formalin fixed, paraffin embedded tissue sections of melanoma. (A) Immunostaining of $P2Y_1$ receptors (pink) with haematoxylin counterstain (purple) in a specimen of melanoma. The melanoma (m) has infiltrated the dermis (d) and has nearly completely replaced it. Note that the staining of basal cells, indicated by arrows, in the stratum basale (sb) of the epidermis (e) in the same section is an internal positive control. Bar = 250 μM. (B) Weak positive staining for $P2Y_2$ receptors of the core of the melanoma (m) with strong positive staining of cells at the proliferating margin (x) as it infiltrates the dermis (d). Bar = 500 μM. (C) $P2Y_2$ receptor staining, indicated by arrows, are present both in melanoma cells (m) and consistently expressed by the basal cells of the epidermis (e) is an internal positive control. Bar = 500 μM. (D) $P2Y_6$ receptors are consistently expressed by melanoma cells, throughout a tissue sample. Bar = 250 μM.

the mRNA is translated into $P2Y_4$ receptor protein, at least in the conditions examined. Previous work has shown that mRNA for many P2Y receptors may be present in various tissues and cells but this does not necessarily reflect functional receptor protein expression [30].

### *3.4. Intracellular calcium levels are altered in response to purinergic compounds*

The concentration of intracellular calcium in the A375 melanoma cells was increased in a dose dependent manner by the addition of ATP (Fig. 4A). In a bath solution containing $Ca^{2+}$ there was a biphasic increase in intracellular calcium levels; an initial peak was followed by a second, sustained lower phase. When ATP was added to cells incubated in a calcium-free bath solution, there was only the initial monophasic rise in intracellular calcium (Fig. 4B). The role of phospholipase C-coupled G proteins in P2Y receptor-mediated signalling [31] was investigated using the phospholipase C specific inhibitor U73122. The ATP mediated rises in intracellular calcium were reduced by pre-incubation for 15 min with U73122 in a dose-dependent manner, whilst the inactive analogue U73343 had minimal effect (Fig. 4C).

The biphasic response to stimulation by ATP is an established feature of metabotropic P2Y receptors [19]. The response to ATP seen is suggestive of an initial rise in intracellular calcium levels due to the mobilization of $Ca^{2+}$ from intracellular stores by

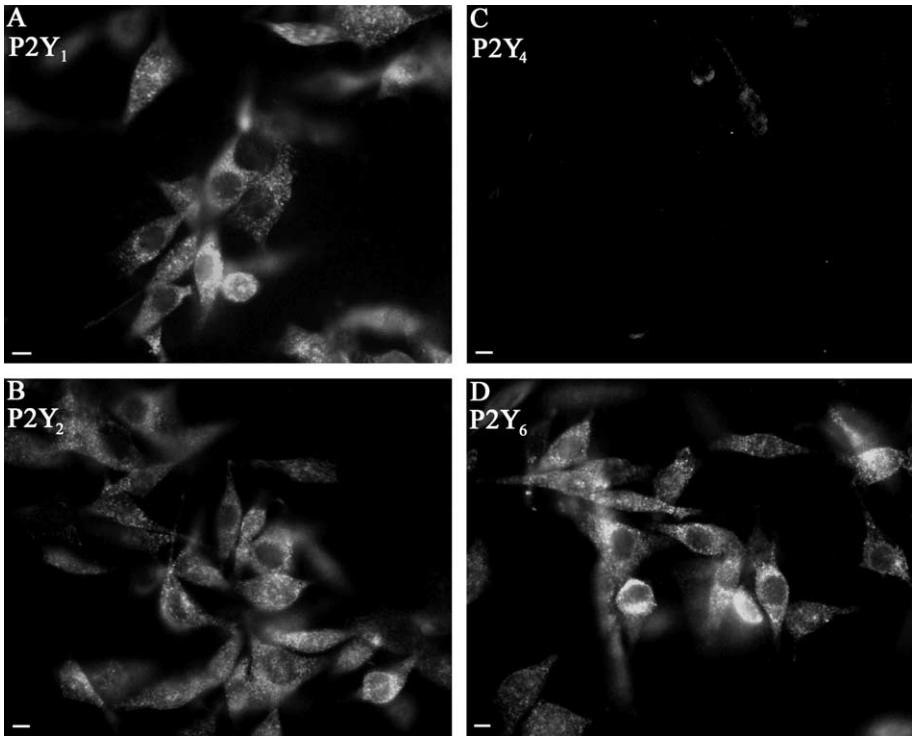


Fig. 2. Immunolocalisation of P2Y receptors in human melanoma cells grown in culture. Fluorescent staining was seen for $P2Y_1$ (A), $P2Y_2$ (B) and $P2Y_6$ (D) receptors, but $P2Y_4$ receptors were not present (C). Bar = 25 μM.

G protein receptors coupled to the phospholipase $C/IP_3$ transduction pathway. This is followed by a second peak in intracellular calcium levels due to calcium-mediated calcium influx across the cell membrane.

To characterize the P2Y receptor subtypes present pharmacologically, changes in intracellular calcium levels were also measured in response to the $P2Y_1$ receptor-selective agonist 2MeSADP, the $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor agonist UTP and the $P2Y_6$ receptor-selective agonist UDP. These compounds were used either alone or following pre-incubation of the cells with the $P2Y_1$ receptor-selective antagonist MRS 2179 and the $P2Y_1$, $P2Y_4$, and $P2Y_6$, receptor antagonist PPADS. Each of the agonists produced an increase in intracellular calcium levels, which was partially reversed by pre-incubation with antagonist in all but one case. At a dose of 10 μM the rank order of potency was 2MeSADP > ATP > UTP > UDP (Fig. 4D). The antagonist MRS 2179 caused a significant reduction in the response to ATP and

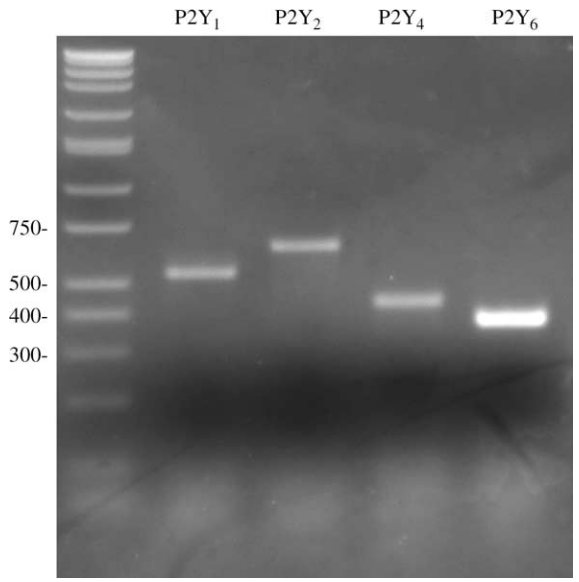


Fig. 3. mRNA for P2Y receptor subtypes $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$, is present in melanoma cells. RT-PCR analysis of total RNA extracted from A375 cells generated fragments of the expected size.

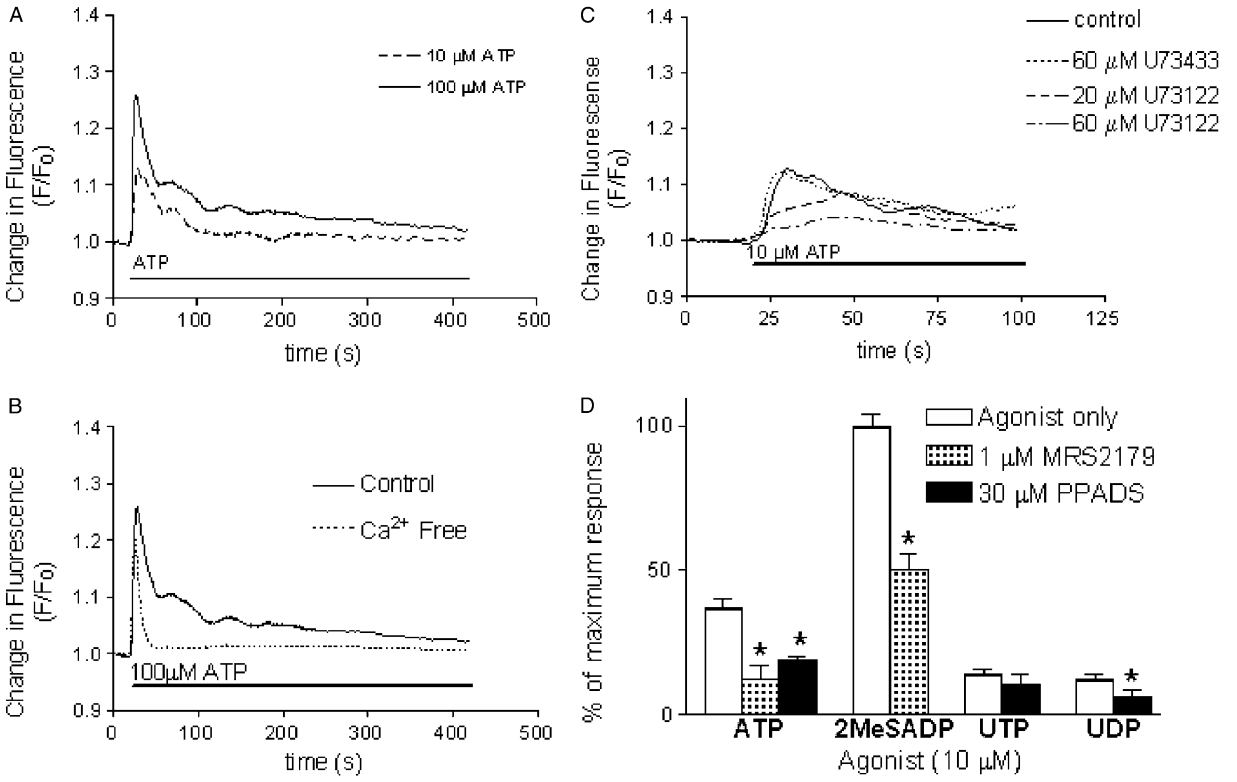


Fig. 4. Intracellular calcium levels, measured using the FLIPR, are raised in A375 melanoma cells after application of extracellular ATP. (A) Dose-dependent biphasic increase in intracellular calcium levels in a bath solution containing $Ca^{2+}$. (B) Rise in intracellular calcium level after application of ATP in a calcium-free bath solution is monophasic. (C) Pre-incubation of cells with the phospholipase C inhibitor U731222 dose-dependently reduced the rise in intracellular calcium in response to ATP. There was minimal change when U73122 was replaced by its inactive analogue U73343. Means of three independent experiments each consisting of three or more wells are shown. (D) P2Y receptor subtype agonists cause reversible increases in intracellular calcium levels. Application of different P2 agonists at equal concentration (10 μM) caused different increases in intracellular calcium. Values plotted are the peak response as a percentage of the maximum response of the agonist with the highest efficacy (2MeSADP). Cells were preincubated with the $P2Y_1$ receptor-selective antagonist MRS 2179 and the $P2Y_1$, $P2Y_4$ and $P2Y_6$ receptor-selective antagonist PPADS for 30 min prior to the addition of agonist. Means ± SEM of three independent experiments each consisting of three to eight wells are shown (*$P<0.05$).

2MeSADP, whilst PPADS caused a significant reduction in the response to ATP and UDP and a minimal, non-significant reduction in the response to UTP. These results suggest the presence of functional $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptor subtypes.

### *3.5. Extracellular nucleotides regulate changes in A375 melanoma cell number*

The proliferative effects of a sustained application of the P2Y receptor agonists ATP, 2MeSADP, UTP and UDP was studied on human melanoma cells using a crystal violet colourimetric assay. The generic, non-specific receptor subtype agonist ATP produced no change in cell number at low concentrations of 1 μM and 10 μM concentration and a decrease in cell number at a high concentration of 100 μM (Fig. 5A). Seventy-two hours after treatment with 100 μM ATP there was a significant reduction of 32% in cell number compared to control ($P<0.05$). The $P2Y_1$ specific agonist 2MeSADP was shown to cause a dose-dependent decrease in cell number compared to control (Fig. 5B) with a 45% decrease in cell number 72 h after treatment with 100 μM 2MeSADP ($P<0.05$), whereas the $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor agonist UTP caused a dose-dependent

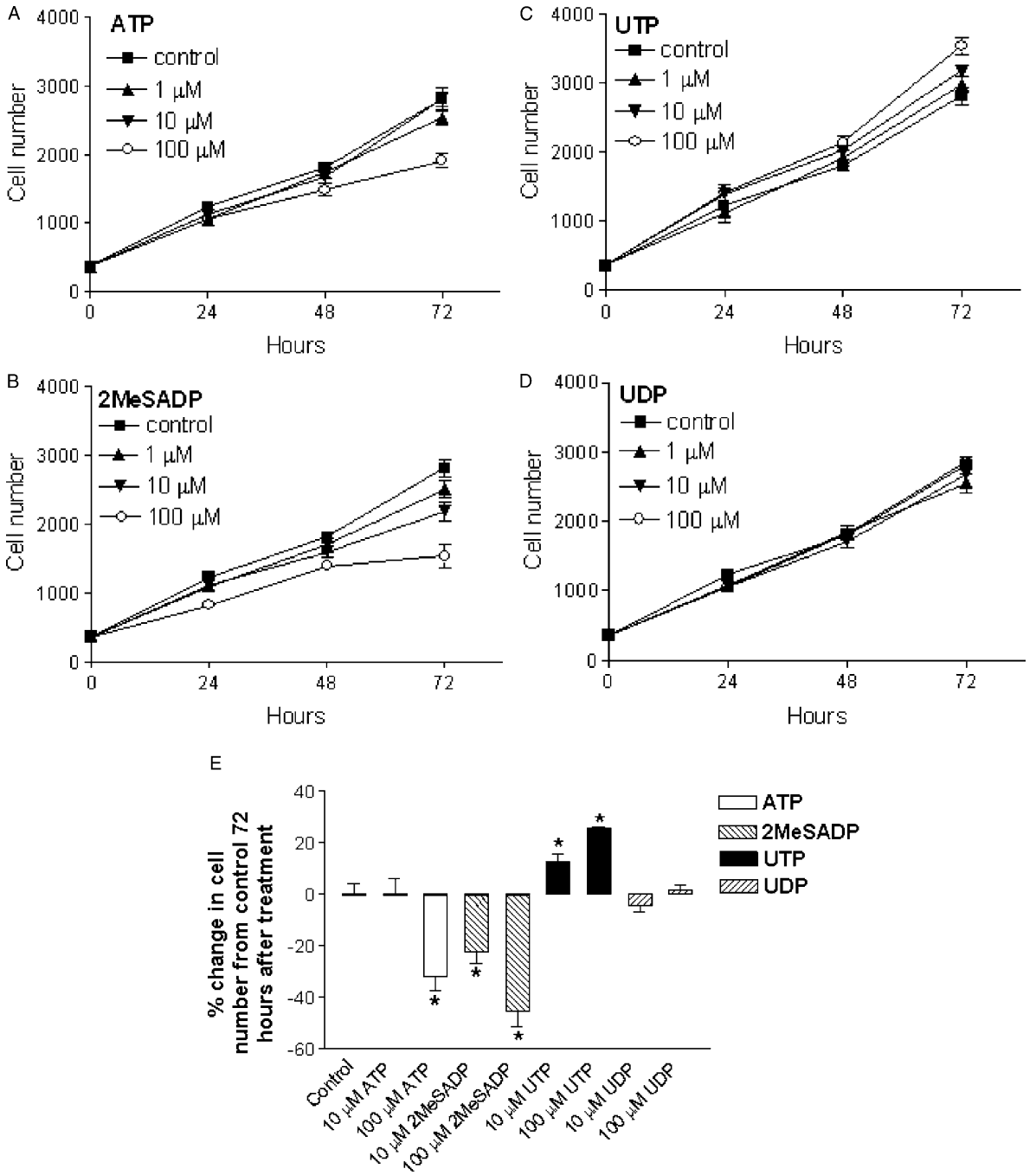


Fig. 5. Effect of extracellular nucleotides on melanoma cell proliferation. (A) ATP at a concentration of 100 μM caused a significant reduction in cell number whereas 1 μM and 10 μM ATP had no effect. (B) 2MeSADP caused a dose-dependent decrease in melanoma cell number. (C) UTP was shown to cause a dose-dependent increase in cell number. (D) Treatment with UDP had no effect on cell number. (E) Summary of results 72 h after treatment with purinergic agonist. Means ± SEM of a minimum of three different experiments each of a minimum of three wells are shown ($n = 9$ or greater), $^*P < 0.05$.

increase in cell number (Fig. 5C). There was an increase of 26% 72 h after treatment with 100 μM UTP ($P<0.05$). The selective $P2Y_6$ receptor agonist UDP showed no effect on changes in cell number (Fig. 5D). These results are summarised in Fig. 5E.

## 4. Discussion

ATP is known to inhibit cancer cell growth in a variety of models and through a number of mechanisms [32]. Attempts have been made to identify the mechanism of action of ATP on cancer cells and the purinergic receptor subtypes involved. Alteration of cancer cell number may be due to mediation of cell proliferation via P2Y receptors [19,33,34], stimulation of differentiation with subsequent inhibition of proliferation via $P2X_5$ receptors [10,35–37] or induction of cell death (apoptosis) via $P2X_7$ receptors [10,38,39]. P2Y receptors have been described in cancer types, other than melanoma, where they mediate changes in cell number. However, P2Y receptors may mediate opposite actions in different cancer types. For instance, $P2Y_2$ receptors have been shown to mediate an increase in cell number in lung cancer [21] and squamous cell carcinoma of the skin [40], but cause a decrease in proliferation in endometrial [41] and colorectal [42] cell lines.

In this paper we provide evidence for functional $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors in the human melanoma A375 cell line at both the cellular and molecular levels and that activation of the $P2Y_1$ receptor causes a decrease in cell number whereas activation of the $P2Y_2$ receptor causes an increase in cell number.

The expression of both P2Y receptor mRNA and protein in the human A375 melanoma cell line was consistent with $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors being elucidated by both techniques. $P2Y_4$ receptor mRNA was expressed, but it was not translated into $P2Y_4$ receptor protein. This pattern of expression was also seen in formalin-fixed, paraffin-embedded specimens of melanoma, which had been excised from patients. $P2Y_1$ and $P2Y_6$ receptors were consistently expressed in these specimens. $P2Y_2$ receptors were also present but were expressed most strongly at the growing edges of the tumour specimens; this is consistent with mediating the proliferation of cells.

The dose-dependent increases in intracellular calcium levels in response to ATP, which was biphasic in a $Ca^{2+}$-containing bath solution but monophasic in a $Ca^{2+}$-free bath solution and inhibited in a dose-dependent manner by U73122 strongly suggests that the action of extracellular nucleotides is mediated by metabotropic P2Y receptors coupled to phospholipase C [19].

Currently, no selective agonists exist for all the purinoreceptors [8] and therefore pharmacological identification relies on the effects of a combination of compounds. ATP itself is active over the entire range of P2Y receptors, although it has different efficacies at each receptor. 2MeSADP is a selective agonist at $P2Y_1$ receptors, UTP is an agonist at $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors and UDP is a selective agonist at the $P2Y_6$ receptor. MRS 2179 is a selective antagonist at the $P2Y_1$ receptor whereas PPADS is an antagonist at the $P2Y_1$, $P2Y_4$ and $P2Y_6$ receptors but not at the $P2Y_2$ receptor.

2MeSADP caused an increase in intracellular calcium which was partially antagonised by MRS 2179; this is strong evidence for the presence of functioning $P2Y_1$ receptors. There was also an increase in intracellular calcium in response to UDP which was partially blocked by PPADS; again this is evidence for the presence of functioning $P2Y_6$ receptors, although little effect on cell proliferation was seen. UTP, active at $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors, also caused a response. As this was not reduced by the antagonist PPADS, an antagonist at $P2Y_1$, $P2Y_4$ and $P2Y_6$ receptors, it is likely the response to UTP is at least partially mediated via the $P2Y_2$ receptor.

The effect of incubation of A375 melanoma cells in the presence of 2MeSADP was a statistically significant dose-dependent decrease in cell number. A reduction in cell proliferation mediated by the $P2Y_1$ receptor has previously been reported in human astrocytoma cancer cells [43]. UTP caused a dose-dependent increase in cell number; the data from the RT-PCR, immunocytochemistry and intracellular calcium studies suggests this increase was mediated by $P2Y_2$ receptors. No change in cell number was seen when cells were incubated with UDP. ATP has an approximately equal affinity at the $P2Y_1$ and $P2Y_2$ receptors, so even though no change in cell number was seen when 1–10 μM ATP was applied, it is possible that ATP is having equal and opposite effects on cell number, mediated through both $P2Y_1$ and

$P2Y_2$ receptors. At a higher dose of 100 μM there was a decrease in cell number though this was not as great as the decrease seen with 2MeSADP, so other receptor subtypes causing an increase in cell number such as the $P2Y_2$ might also still be activated.

Four of the currently described P2Y receptors have been studied in detail in this paper. As more P2Y receptor subtypes are described and tools for their study, such as specific antibodies, become available it is possible that other P2Y receptors may be shown to have a role in regulation of melanomas, which may be linked to other intracellular messenger systems. This is suggested by the antagonists PPADS, MRS 2179 and U73122 incompletely blocking the effect of the purinergic agonists used.

In summary, we have shown for the first time, there are functional P2Y receptors in human melanomas which regulate cell proliferation. $P2Y_1$ receptors cause a decrease in cell number whereas $P2Y_2$ receptors cause an increase in cell number. Functional $P2Y_6$ receptors are also present but were shown not to have an effect on cell number, suggesting they may play another role in cell regulation. Targeting purinergic receptors may be a putative treatment for malignant melanoma.

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