Adenosine A_{2A} and A_{2B} receptor expression in neuroendocrine tumours: potential targets for therapy

A. Kalhan · B. Gharibi · M. Vazquez · B. Jasani ·
J. Neal · M. Kidd · I. M. Modlin · R. Pfragner ·
D. A. Rees · J. Ham

Received: 14 October 2011 / Accepted: 4 November 2011 / Published online: 27 November 2011
© Springer Science+Business Media B.V. 2011

Abstract The clinical management of neuroendocrine tumours is complex. Such tumours are highly vascular suggesting tumour-related angiogenesis. Adenosine, released during cellular stress, damage and hypoxia, is a major regulator of angiogenesis. Herein, we describe the expression and function of adenosine receptors (A₁, A_{2A}, A_{2B} and A₃) in neuroendocrine tumours. Expression of adenosine receptors was investigated in archival human neuroendocrine tumour sections and in two human tumour cell lines, BON-1 (pancreatic) and KRJ-1 (intestinal). Their function, with respect to growth and chromogranin A secretion was carried out in vitro. Immunocytochemical data showed that A_{2A} and A_{2B} receptors were strongly expressed in 15/15 and 13/18 archival tumour sections. Staining for A₁ (4/18) and A₃ (6/18) receptors was either very weak or absent. In vitro data showed that adenosine stimulated a three- to fourfold increase in cAMP levels in BON-1 and KRJ-1 cells. The non-selective adenosine

receptor agonist (adenosine-5'-N-ethylcarboxamide, NECA) and the A_{2A}R agonist (CGS21680) stimulated cell proliferation by up to 20–40% which was attenuated by A_{2B} (PSB603 and MRS1754) and A_{2A} (SCH442416) receptor selective antagonists but not by the A₁ receptor antagonist (PSB36). Adenosine and NECA stimulated a twofold increase in chromogranin A secretion in BON-1 cells. Our data suggest that neuroendocrine tumours predominantly express A_{2A} and A_{2B} adenosine receptors; their activation leads to increased proliferation and secretion of chromogranin A. Targeting adenosine signal pathways, specifically inhibition of A₂ receptors, may thus be a useful addition to the therapeutic management of neuroendocrine tumours.

Keywords Adenosine · A_{2A} adenosine receptor · A_{2B} adenosine receptor · Human neuroendocrine tumours · Cell proliferation · Chromogranin A

A. Kalhan · B. Gharibi · M. Vazquez · D. A. Rees · J. Ham (✉)
Centre for Endocrine and Diabetes Sciences, School of Medicine,
Cardiff University,
Heath Park,
Cardiff CF14 4XN, UK
e-mail: wmdjh@cardiff.ac.uk

B. Jasani · J. Neal
Department of Pathology, School of Medicine, Cardiff University,
Cardiff CF14 4XN, UK

M. Kidd · I. M. Modlin
Department of Surgery, Yale University School of Medicine,
New Haven, CT, USA

R. Pfragner
Institute of Pathophysiology and Immunology, Centre for
Molecular Medicine, Medical University of Graz,
Graz, Austria

Introduction

Neuroendocrine tumours (NETs) are a heterogeneous group of neoplasms of unknown aetiology which vary broadly both in their clinical presentation and biological behaviour. Such tumours are widely distributed and originate, as their name suggests, from neuroendocrine cells throughout the body. These tumours are often characterised by excessive secretion of hormones and biogenic amines. In a local setting, surgery is the treatment of choice for metastatic disease and clinical management is aimed at reducing hormone-associated symptoms. The mainstay of treatment for patients with metastatic disease consists of long-acting somatostatin analogues which improve quality of life by controlling symptoms related to hormonal hypersecretion [1]. Of the somatostatin analogues, octreotide has been

shown to stabilise tumour progression in some well-differentiated NETs [2] and SOM230 (which displays broader receptor affinity) possesses anti-angiogenic activity [3]. In vitro experiments also show that somatostatin can induce morphological changes in neuroendocrine cells [4]. However, with these treatments there is virtually no tumour shrinkage in the majority of patients with metastatic disease.

The increased vascularity of endocrine tumours [5] suggests that agents targeting vascular endothelial growth factor (VEGF) and other angiogenic pathways might have value in the treatment of patients with NETs. VEGF levels in NET tissue show an inverse correlation with patient survival [6] suggesting that VEGF expression and the mediation of angiogenesis are associated with human NET growth. There are several clinical trials involving pharmaceutical agents (Bevacizumab and Sunitinib) that are aimed at targeting VEGF secretion or inhibition of the VEGF receptor [7]. Hypoxia is a major regulator of angiogenesis, mediated in part via up-regulation of hypoxia-inducible factor (HIF)-1 α [8]. The purine nucleoside adenosine, released from hypoxic tissues, appears to be a key mediator in this pathway, accounting for up to 70% of hypoxia-induced angiogenesis in some situations [9–11]. Adenosine mediates its actions through four adenosine receptors, A₁, A_{2A}, A_{2B} and A₃, which are differentially expressed in different tissues [12]. Therapeutic modulation of adenosine levels or signalling could therefore provide a basis for the treatment of many cancers [13–15]. In this study, we show that A_{2A} and A_{2B} receptors are the dominant adenosine receptors in human NET cell lines and in archival human NET tissue. Our in vitro data indicate that activation of these receptors can mediate cell proliferation and chromogranin A secretion. Thus the specific inhibition of A₂ signal pathways may be useful for the therapeutic management of NETS.

Materials and methods

Materials Cell culture materials and reagents were obtained from Lonza (Wokingham, UK), Biosera (Ringmer, UK), Fisher Scientific (Loughborough, UK), Invitrogen (Paisley, UK) and Sigma–Aldrich (Poole, UK). Specific polyclonal antisera to human A₁ and A_{2A} receptors were from Abcam (Cambridge, UK) and affinity-purified antisera to the A_{2B} and A₃ receptors were from Alpha Diagnostic International (San Antonio, USA). Adenosine receptor agonists and antagonists were obtained from Tocris (Avonmouth, UK). Vectastain Elite ABC kits (Vector Laboratories, Peterborough, UK) were used for immunocytochemistry. Molecular biology reagents, except TRIzol (from Invitrogen), were obtained from Promega, (Southampton, UK). Western blotting reagents were from GE Healthcare (Chalfont St. Giles, UK).

Adenosine receptor immunocytochemistry in human NETS Eighteen representative blocks of NETs (eight ileocaecal, four pancreatic, four appendiceal, one ovarian and one liver metastasis) were retrieved from the histopathology tissue archives at the University Hospital of Cardiff, Wales. For this, ethical approval was obtained from the local research ethics committee (approval number 07/WSE02/1). H and E sections (haematoxylin and eosin) from each of these blocks were scanned using Mirax Scan (Carl Zeiss, Gottingen, Germany) and the digital images viewed by a trained pathologist to identify tumour bearing tissue. Two or three 1.5-mm tissue cores were punched out from each of these areas using a microarray device to prepare two tissue microarrays containing tissue cores from nine patients each. Serial sections were cut from each of the resulting tissue microarray blocks and immunostained for adenosine receptors using an automated Shandon Varistain Gemini programme (Fisher Scientific, Loughborough, UK) coupled to the DAKO Autostainer Plus (DAKO UK Ltd, Ely, Cambridgeshire, UK) procedure based on Vectastain ABC Elite kits (Vector Laboratories, Peterborough, UK). Staining was conducted using polyclonal antibodies to A₁, A_{2A}, A_{2B} and A₃ adenosine receptors at optimal dilutions of 1:200, 1:400, 5 μ g/ml and 1:400, respectively, and applied for 1 h at room temperature. Sections were then incubated for 30 min in the appropriate second antibody coupled to biotin and then visualised with diaminobenzidine (5 min). Nuclei were counter-stained with haematoxylin. For negative controls, non-immune mouse serum (at 1:200 dilution) was used. Immunostaining was reviewed independently by two pathologists (JN and BJ) and scored to be either negative (0), weak (1+), moderate (2+) or strong (3+).

Cell culture BON-1 cells (a generous gift from Dr. Ursula Plockinger, Interdisziplinäres Stoffwechsel-Centrum, Charité-Universitätsmedizin Berlin, Campus Virchow-Klinikum, Berlin, Germany) were cultured in DMEM/Ham's F12 (50% v/v) and 10% (v/v) FBS (foetal bovine serum). KRJ-I cells [16–18] were cultured in Ham's F12 and 10% (v/v) FBS.

RT-PCR Total cellular RNA was prepared using TRIzol reagent and treated with RQ1 ribonuclease-free deoxyribonuclease. RNA (0.2 μ g) was reverse transcribed using oligodeoxythymidilic acid [oligo(dT)₁₅] for 1 h at 37°C and the cDNA generated was subjected to PCR amplification using primers specific for human adenosine receptors. Primer sequences (shown in Table 1) were designed using the Primer 3 software programme and gene sequences obtained from GenBank. For the PCR reaction 30–40 cycles were carried out as follows: 94°C, 65°C and 72°C for 30 s, 1 min and 1 min, respectively, and a final extension step of 72°C for 10 min. Amplified products were electrophoresed in 2% (w/v) agarose and visualised with ethidium bromide.

Table 1 PCR primer sequences and amplicon sizes

Gene	Forward	Reverse	Base pairs, bps
A1R	GTCCTCATCCTCACCCAGAG	CAGATTGTTCCAGCCAAACA	189
A2aR	TCTTCAGTCTCCTGGCCATC	TCCAACCTAGCATGGGAGTC	156
A2bR	CTCCATCTTCAGCCTTCTGG	GGAATGGAGTCAATCCGATG	152
A3R	GGGCATCACAATCCACTTCT	AGGGCCAGCCATATTCTTCT	171
CD73	ACCAGGGCACTATCTGGTTC	AGTGGCTCGATCAGTCCTTC	133
Adenosine deaminase	GACCCGCTCATCTTCAAGTC	GGTCGAGAAGCTCCCTCTTT	151
Adenosine kinase	TCACCCAAGGGAGAGATGAC	ATAGTGGCCAGCACGGATAC	185
Chromogranin A	AGAAACACAGCGTTTTGAAGA	TCTGCCTCCTTGAATCCTCT	136

Quantitative (q) RT-PCR, which is much more sensitive than conventional PCR, was also used to assess adenosine receptor expression in BON-1 and KRJ-1 cells. qRT-PCR was performed on the MX3000P thermal cycler system (Stratagene, Cambridge, UK). The reaction mix was: 12.5 µl Platinum SYBR Green qPCR Supremix-UDG (Invitrogen), 1 µl cDNA, 1 µl (10 nmol) forward and reverse primers and 10.5 µl nuclease-free water. The thermal profile was 1 cycle (95°C 10 min), 40 cycles (95°C 30 s, 60°C 1 min, 72°C 30 s) and 1 cycle (95°C 1 min, 60°C 30 s, 95°C 30 s). All four genes were assessed in the same sample, performed in triplicate and in three separate experiments.

Western blotting analysis Western blotting analysis was used to demonstrate expression of adenosine receptor (A_1 , A_{2A} , A_{2B} and A_3) protein in BON-1 and KRJ-I cells. BON-1 monolayers and pelleted KRJ-I cells were lysed in 200 µl RIPA buffer containing 1 mM sodium orthovanadate, 0.1 mg/ml PMSF and chymostatin, leupeptin, antipain and pepstatin A (all at 10 µg/ml). Lysates after centrifugation were stored at −80°C. Aliquots (15 µl) from each treatment were mixed with an equal volume of electrophoresis buffer, boiled and electrophoresed in 10% (w/v) polyacrylamide. Proteins were transferred onto PVDF membranes and incubated overnight at 4°C with antisera to adenosine receptors at a concentration of 1:1,000, except for the A_{2A} receptor ($A_{2A}R$) antiserum which was used at 1:2,000. Secondary anti-rabbit IgG conjugated to horseradish peroxidase, at 1:5,000, was applied for 1 h at room temperature and proteins were visualised with ECL Plus reagent.

cAMP BON-1 and KRJ-I cells were each plated out at a density of 0.1×10^6 cells/well into untreated (BON-1) 48-well multidishes or dishes that had been pre-coated with poly-D-lysine (70–150 Kd, 0.1 mg/ml) (KRJ-1). After 1–2 days in culture, cells (replicates $\times 4$) were gently washed in serum-free culture medium and incubated for 15 min with adenosine, adenosine-5'-N-ethylcarboxamide (NECA) and CGS21680 at a concentration range of 10 nM to 0.1 mM

in the presence of 0.1 mM R_o 20-1724 (cyclic nucleotide phosphodiesterase inhibitor). Cells were solubilised in 0.2 ml 0.1 M HCl and stored at −20°C; the acid was removed by rotary vacuum evaporation and the residues dissolved in 0.5 ml cAMP assay buffer (50 mM sodium acetate, 0.25% w/v bovine serum albumin, pH 5.2). cAMP measurements were carried out as previously described [19].

Cell growth Growth of BON-1 and KRJ-I cells was determined using the MTS proliferation assay kit from Promega (Southampton, UK). Briefly, BON-1 (2,000/well) and KRJ-I (25,000/well) cells were plated into 96-well multiwell plates in a volume of 0.05 ml, respectively, in DMEM/Ham's F12 1% (v/v) FBS and Ham's F12 1.25% (v/v) FBS. After an overnight incubation, replicate wells ($\times 8$) were treated with adenosine, NECA and CGS21680 at a concentration range 10 nM to 10 µM for 48 and 72 h. At the end of each time point, 0.02 ml MTS reagent was added and the formazan product measured by absorbance at 490 nm after 1 or 4 h (KRJ-I cells). A blank absorbance reading (culture medium alone) was subtracted from each value. The quantity of formazan produced is directly proportional to the number of living cells in culture. In some experiments, cells were incubated with adenosine receptor antagonists, PSB36 (A_1), SCH442416 (A_{2A}), PSB603 and MRS1754 (A_{2B}), in the presence of agonist for 3 days.

Direct cell counting was also carried out by image cytometry using the Cellometer Auto T4 counter (Nexcelom Bioscience LLC, Lawrence, MA, USA). Images of cells in a disposable counting chamber (very similar to a haemocytometer) are taken and analysed using the attached software from which cell number per milliliter is calculated.

Chromogranin A BON-1 cells (0.2×10^6 cells/well) were plated out into 24-well multidishes and allowed to become confluent. Replicate wells ($\times 4$) were treated with the AR agonists, adenosine, NECA and CGS21680 over the concentration range 10 nM to 0.1 mM for 1 h at 37°C in serum-free DMEM/Ham's F12. Supernatants were collected

Table 2 Summary of adenosine receptor immunostaining in archival human NETs. (staining intensity was graded 0 (absent), 1+ (weak), 2+ (moderate) and 3+ (strong))

Receptor	Appendix	Midgut	Pancreas	Miscellaneous	Total	Staining grade
A ₁	1/4	1/8	1/4	1/2	4/18	0–1+
A _{2A}	4/4	8/8	3/3	3 IM ^a	15/15	1–3+
A _{2B}	4/4	4/8	4/4	1/2	13/18	2–3+
A ₃	2/4	1/8	2/4	1/2	6/18	0–1+

^a Insufficient material for analysis

and stored at -20°C prior to assay for chromogranin A using the radioimmunoassay kit from BioSource (Nivelles, Belgium), cat. no. KIPERB321. Similar experiments were carried out with KRJ-1 cells plated into poly-D-lysine-coated plates; these were left for 3 days and incubated with adenosine receptor agonists for 1–8 h.

Statistical analysis Experiments were performed three to five times (n) with up to eight replicates for each treatment. Results were analysed with INSTAT 3 software (GraphPad Software, La Jolla, USA) and expressed as mean $\pm$ SEM and compared by ANOVA and the Tukey comparison test. $P < 0.05$ is deemed to be significant.

Results

Expression of adenosine receptors in archival human NETS Adenosine receptor expression in human NETS patients was investigated with immunocytochemistry on a limited number of archival samples. A summary of the

staining is shown in Table 2. Staining for A₁ and A₃ receptors were either absent or only weakly positive and only in a minority (4/18 and 6/18, respectively) of the tumours. In contrast, the A_{2A}R was expressed in all of the tumours tested (15/15, there was insufficient material in three sections) whilst the A_{2B} receptor (A_{2B}R) was positive in most (13/18) of the tumours; the staining intensity although variable was overall much stronger than that for the A₁ and A₃ receptors. In addition, tumours that were negative for A_{2B}R were restricted to the small bowel where 4/5 tumours showed little or no staining, on the other hand, all of these tumours stained heavily for the A_{2A}R. Figure 1a and b respectively show strong immunostaining for A_{2A} and A_{2B} receptors in a pancreatic tumour and strong A_{2A}R but little or no A_{2B}R staining in a small bowel tumour (Fig. 1d–e). There was little cellular staining in the absence of antisera as shown in Figure 1c and f. Closer examination of the slides also showed apparent staining for both A_{2A} and A_{2B} receptors in the connective tissue, this is likely to be non-specific staining as it was also apparent in the negative controls. The three staining images shown for each

Fig. 1 A_{2A} and A_{2B} receptor immunostaining in a human pancreatic and a small bowel tumour. Immunostaining for the A_{2A}R (a and d), the A_{2B}R (b and e) and with non-immune sera (c and f) in a pancreatic (a–c) and a small bowel tumour (d–f). Note the absence of cellular staining for the A_{2B}R in e. Non-specific staining of connective tissue was also noted, even in control cells

tumour type are from the same tumour. Archival placenta and stomach tissues were used as positive controls for adenosine receptors (not shown).

Expression of adenosine receptors in BON-1 and KRJ-1 cells Adenosine receptor gene expression in BON-1 and KRJ-1 cells was determined with PCR, Fig. 2a shows that all four adenosine receptors were present in both cell lines together with adenosine deaminase, adenosine kinase and CD73 (ecto-5'-nucleotidase), enzymes that are involved in the direct synthesis and metabolism of adenosine. Expression of A₁R and A₃ receptor (A₃R) in both KRJ-I and BON-1 cells appeared to be particularly weak and detection was only possible when the number of PCR cycles was increased to 40. DNA sequencing however did confirm their presence (data not shown).

qRT-PCR was also used to confirm adenosine receptor gene expression; in the same cDNA sample cycle threshold (Ct) values for BON-1 cells and KRJ-I cells were, respectively, A₁R 34±2, 36±3; A_{2A}R 21±2, 21±1; A_{2B}R 21±2, 26±2 and A₃R 35±3, 31±3. The lower the Ct value, the higher the expression of mRNA and an increase of 1 equates to a halving of expression. Although it is not valid to compare Ct values from different genes, these data also indicate that A_{2A}R and A_{2B}R are the dominant receptors in BON-1 and KRJ-I cells. Western blotting analysis showed that the proteins for all four receptors were also expressed

Fig. 2 Adenosine receptor and adenosine metabolic enzyme expression in BON-1 and KRJ-I cells. **a** RT-PCR for adenosine receptor and adenosine metabolic enzyme mRNA in KRJ-I and BON-1 cells, AD-adenosine deaminase, AK-adenosine kinase, CD-CD73 (ecto-5'-nucleotidase). The expected amplicon sizes are shown in Table 1 and the numbers on the left indicate the number of base pairs. **b** Western blotting analyses of adenosine receptors in BON-1 (B) and KRJ-1 (K) cells; the positions of the marker proteins are indicated

in both cell lines (Fig. 2b), although it is not possible to compare band intensities from different antisera, this data, as with gene expression, could also suggest that the A_{2A}R and A_{2B}R are the dominant receptors.

In order to confirm which receptors had the highest expression, we carried out functional cAMP measurements as A₂ receptors primarily stimulate cAMP and A₁ and A₃ receptors inhibit cAMP. Figure 3 shows that adenosine, in a dose-related manner, stimulated endogenous cAMP levels in both KRJ-I cells (Fig. 3a) and BON-1 cells (Fig. 3b) by up to three- to fourfold and ED₅₀ of 5–10×10⁻⁷ M. Interestingly, the stable non-selective adenosine receptor agonist, NECA, was most potent (up to sixfold) in BON-1 cells (Fig. 3b) whereas the selective A_{2A}R agonist, CGS21680, was most potent in KRJ-I cells (up to seven fold; Fig. 3a).

Effects of adenosine on growth of BON-1 and KRJ-1 cells Our data show that BON-1 and KRJ-1 cells express predominantly A_{2A} and A_{2B} receptors, we thus investigated

Fig. 3 Effects of adenosine receptor agonists on intracellular cAMP accumulation in **a** KRJ-I and **b** BON-1 cells. Replicate cultures were incubated with adenosine receptor agonists and the phosphodiesterase inhibitor, R_o 20–1724 for 15 min; cells were extracted into HCl, vacuum dried and reconstituted in assay buffer for cAMP radioimmunoassay. Values shown are percentage changes (mean±SEM) from five experiments with four replicates for each treatment. **P*<0.05, ***P*<0.01, ****P*<0.001 when compared with untreated (basal) cells

if activation of these receptors can influence cell growth. Using the MTS proliferation assay, a single dose of NECA and CGS21680 and incubation for 3 days, stimulated growth of BON-1 and KRJ-I cells in a dose-related manner by 20–40% (Fig. 4a, b). The growth effects of NECA and CGS21680 were markedly greater in BON-1 than in KRJ-I cells. When the actual number of cells were physically counted similar results were obtained (20–45% stimulation in BON-1 cells and 10–15% in KRJ-I cells after three days of exposure to adenosine receptor agonists; Fig. 4c).

Selective adenosine receptor antagonists were used to confirm which of the receptors were involved in the mediation of growth; these experiments were carried out on BON-1 cells as these cells showed the largest changes in growth. When PSB603 (A_{2B} R antagonist) was incubated along with NECA, the growth stimulating effects of the latter were attenuated in a dose-related fashion (Fig. 5a). SCH 442416 (A_{2A} R antagonist) and PSB36 (A_1 R antagonist) at the same molar concentrations did not inhibit NECA-stimulated growth (Fig. 5a). We also used a second A_{2B} R antagonist, MRS1754; this also attenuated NECA-stimulated growth in a dose-related manner (Fig. 5b). Although SCH 442416 failed to inhibit NECA-mediated growth of BON-1 cells, it did

in fact reduce growth stimulation by the A_{2A} R agonist, CGS21680 (Fig. 5c). These data together confirm that A_{2A} and A_{2B} receptors are both involved in adenosine-mediated cell growth. As these receptors act through cAMP we also showed that dibutyryl cAMP ($115\pm4\%$ at $1\text{ }\mu\text{M}$) and forskolin ($140\pm5\%$ at $0.1\text{ }\mu\text{M}$) stimulated cell growth in BON-1 cells, similar but less prominent data were seen in KRJ-1 cells.

Effect of adenosine receptor agonists on chromogranin a secretion from BON-1 cells Adenosine, NECA and CGS21680 stimulated a twofold increase in chromogranin A secretion from BON-1 cells over a 1-h incubation period (Fig. 6a–c). As expected, NECA and CGS21680 were considerably more potent than adenosine itself and probably reflect the labile nature of adenosine and its short half-life in culture media. These data are consistent with the findings observed with cAMP and cell growth (Figs. 3 and 4). Chromogranin A however was not measurable in the conditioned media from KRJ-I cells (data not shown) even after 8-h exposure to agonists; the mRNA and protein were detectable by RT-PCR (unpublished observation-J. Ham) and immunofluorescence, respectively [18].

Fig. 4 Growth effects of adenosine receptor agonists on BON-1 and KRJ-I cells as determined using the MTS proliferation assay and cell number counting. BON-1 (a) and KRJ-1 (b) cultures (eight replicates in duplicate) were incubated with varying concentrations of NECA or CGS21680 (single treatment) for 3 days; MTS solution was then added and the absorbance reading taken at 490 nm. Values shown are percentage changes (mean±SEM) from three experiments. c BON-1

and KRJ-I cells were incubated with the indicated concentrations (single treatment) of adenosine receptor agonists for 3 days; cells were harvested and numbers counted with the Cellometer cell counter. Values shown are mean±SEM×10⁶ of four experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ when compared with untreated (basal) cells

Fig. 5 Effect of adenosine receptor antagonists on NECA and CGS21680 mediated growth of BON-1 cells. BON-1 cells were incubated with NECA (**a** and **b**) or CGS21680 (**c**) for 3 days in the presence of varying concentrations of adenosine receptor antagonists: MRS1754 and PSB603 ($A_{2B}R$ antagonists), SCH442416 ($A_{2A}R$

antagonist) and PSB36 (A_1R antagonist). Values shown are percentage changes (mean $\pm$ SEM) of three to four experiments. *** P <0.001 when compared with untreated (basal) cells. # P <0.05, ## P <0.01 when compared with agonist only treated (0) cells

Discussion

The aetiology of NETS remains unclear and therapeutic management is focussed either on surgery or, if resection is not possible, then somatostatin analogues are the drugs of choice. More recent treatments have been based either on mTOR (mammalian target of rapamycin) or VEGF and related receptors. Since adenosine is a mediator of VEGF release acting through HIF-1 α , we explored the expression and function of adenosine receptors in NETs. A number of other studies in other cancers suggest there could be a possible role for adenosine in the development and survival of NETs. For example adenosine has been shown to modulate the migration of colon cancer cells via an HIF-1 α /VEGF/IL (interleukin)-8-dependent mechanism [14, 20] and A_3R agonism inhibits proliferation or stimulates apoptosis in breast cancer and colon carcinoma cells [21–23]. Agonism at the A_1R has also been reported to induce apoptosis of human colonic cancer cells [24] whereas other

studies suggest that the A_1R in breast carcinoma cells mediate cell cycle progression and cell proliferation [25].

The presence of adenosine receptors on NET cells has by and large been unexplored; our data with archival material show that A_{2A} and A_{2B} receptors are dominant with much weaker expression of the A_1 and A_3 receptors. Although the numbers of samples investigated were limited, our results and those of others [26] using the human BON-1 and KRJ-1 cell lines concur with these findings. The qRT-PCR suggests that there may be similar expression of $A_{2A}R$ in BON-1 and KRJ-1 cells but significantly higher ($\times 30$) expression of $A_{2B}R$ in BON-1 cells. This was reflected in a greater cAMP stimulation in response to NECA in BON-1 cells but a greater effect of CGS21680 in KRJ-1 cells (Fig. 3). Similarly, NECA showed a greater growth-promoting effect than CGS21680 in BON-1 cells whereas both compounds were equally active in KRJ-1 cells (Fig. 4). Interestingly, 4/5 small bowel tumours were negative for the $A_{2B}R$, the significance of this is however

Fig. 6 Effects of adenosine receptor agonists on chromogranin A secretion from BON-1 cells. BON-1 cultures were treated with varying concentrations of adenosine receptor agonists, adenosine, NECA and CGS21680 for 1 h as indicated. Secreted chromogranin A was measured by radioimmunoassay. Values shown are percentage changes (mean±SEM) from three experiments. * $P<0.05$ when compared with untreated (basal) cells

unclear. We also do not know if our findings represent over expression of A_{2A} and A_{2B} receptors or under expression of A_1 and A_3 receptors. Whether adenosine receptors on tumour cells are activated depends on the microenvironment surrounding the tumour; large tumours in particular possess a hypoxic core with high concentrations of extracellular ATP and adenosine [27, 28]. In animals with experimentally induced tumours, ATP levels have been reported to be greater than 100 μ M in tumour tissue and essentially undetectable in healthy tissues [28]. The half-life of ATP is however very short and its degradation product, adenosine has also been shown to accumulate in tumours [27, 29] where it can stimulate growth and angiogenesis and mediate immunosuppression. It is thus likely that the

concentrations of adenosine seen in the microenvironment of tumours would be sufficient to activate adenosine receptors including the low affinity, $A_{2B}R$.

The $A_{2B}R$ has been shown, in gene expression profiling data, to be expressed at a higher level in some cancer tissues and may thus play a role in cancer progression [30–32]. Our data showing that A_2 receptors mediate growth of BON-1 and KRJ-1 neuroendocrine cells would support these findings. The proinvasive action of adenosine in human colon cancer cells is also mediated through the $A_{2B}R$ and co-activation of the other adenosine receptor subtypes [33]. On the other hand, activation of $A_{2B}R$ on immune cells and the associated production of VEGF can also promote tumour growth [34]. There is also evidence, using adenosine receptor-deficient animals, that cancerous cells which express the $A_{2A}R$ and possibly the $A_{2B}R$ may be more resilient to the anti-tumour effects of T lymphocytes [35, 36]. Adenosine may itself be beneficial for tumour cells as it can suppress the activity of inflammatory cells and also stimulate the local production of endothelial cells and subsequent angiogenesis. The actions of the $A_{2A}R$ can be further complicated via possible synergism with dopamine D2 receptors [37]; whether this actually occurs in NET cells is not known but D2 receptors are co-expressed with somatostatin 2 and 5 receptors [38].

Adenosine, itself, could also be directly involved in tumour growth, as targeted inhibition of the enzyme, CD73 (the enzyme that dephosphorylates AMP to adenosine), has been shown to inhibit breast cancer growth and metastasis [39]. These data suggest that the accumulation of adenosine stimulates breast cancer cell growth which is further supported by the report that the $A_{2A}R$ agonist, CGS21680, stimulates proliferation of MCF-7 cells [40]. In our growth experiments, the observed changes were small, typically 30–40% in the presence of NECA or CGS21680 for BON-1 cells. These values are in line with those reported for the growth effects of 30 μ M adenosine on HT-29 colorectal carcinoma and MCF-7 breast carcinoma cells over an incubation time of 8–10 days [41, 42]. As for HT-29 cells [41], prolonged agonist exposure (10–12 days) could perhaps lead to markedly greater growth effects in our experiments. The affinities of adenosine for the different adenosine receptors are quite different; A_1 and A_{2A} being essentially high affinity (nanomolar) and the A_{2B} and A_3 receptors low affinity (micromolar) [12]. Thus, the concentrations of the adenosine receptor agonists and antagonists used in our experiments were fairly high but necessary to activate the $A_{2B}R$ expressed on both BON-1 and KRJ-1 cells.

Plasma chromogranin A levels has been used as a diagnostic and prognostic indicator for NETS, particularly those of intestinal or pancreatic origin and one of the aims of therapeutic management is to lower hormone-associated symptoms [7, 43, 44]. Our data shows that activation of A_2

receptors induces a rapid release of chromogranin A as well as stimulating cell growth. This stimulated secretion of chromogranin A is likely to be from preformed peptide stores rather than due to increased mRNA translation as we have data to show that message transcripts in BON-1 cells were unchanged after exposure to 1 μ M NECA for up to 24 h (data not shown). A₂ receptor-mediated chromogranin A secretion is probably via the activation of cAMP, this is consistent with the presence of a consensus cAMP element in the promoter sequence of the human chromogranin A gene [45].

In summary, we have shown that NETS express predominantly A_{2A} and A_{2B} adenosine receptors and adenosine acting via these receptors mediates cell growth and chromogranin A secretion. Selective inhibition of adenosine receptors or its signal pathways could serve as additional targets adjunct to the current therapeutic targets, somatostatin and VEGF.

Acknowledgements The authors thank J. Stott for carrying out immunostaining of the human NET samples and financial support from Cardiff University (School of Medicine).

Conflicts of interest None.

References

- Rubin J, Ajani J, Schirmer W, Venook AP, Bukowski R, Pommier R et al (1999) Octreotide acetate long-acting formulation versus open-label subcutaneous octreotide acetate in malignant carcinoid syndrome. *J Clin Oncol* 17:600–606
- Arnold R, for the PROMID Study Group (2009) Placebo-controlled, double-blind, prospective, randomized study of the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors: a report from the PROMID study group. *J Clin Oncol* 27(28):4656–4663. doi:10.1200/JCO.2009.22.8510, ASCO GI Cancers Symposium
- Adams RL, Adams IP, Lindow SW, Zhong W, Atkin SL (2005) Somatostatin receptors 2 and 5 are preferentially expressed in proliferating endothelium. *Brit J Cancer* 92:1493–1498
- Saras J, Gronberg M, Stridsberg M, Oberg KE, Janson ET (2007) Somatostatin induces rapid contraction of neuroendocrine cells. *FEBS Lett* 581:1957–1962
- Oberg K (1994) Biology, diagnosis and treatment of neuroendocrine tumors of the gastrointestinal tract. *Curr Opin Oncol* 6:441–451
- Zhang J, Jia Z, Li Q, Wang L, Rashid A, Zhu Z et al (2007) Elevated expression of vascular endothelial growth factor correlates with increased angiogenesis and decreased progression-free survival among patients with low-grade neuroendocrine tumours. *Cancer* 109:1478–1486
- Modlin IM, Moss SF, Oberg K, Padbury R, Hicks RJ, Gustafsson BI et al (2010) Gastrointestinal neuroendocrine (carcinoid) tumours: current diagnosis and management. *Med J Australia* 193:46–52
- Bruick RK (2003) Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. *Gene Dev* 17:2614–2623
- Adair TH (2005) Growth regulation of the vascular system: an emerging role for adenosine. *Am J Physiol-Reg I* 289:R283–R296
- Merighi S, Benini A, Mirandola P, Gessi S, Varani K, Leung E et al (2006) Adenosine modulates vascular endothelial growth factor expression via hypoxia-inducible factor-1 in human glioblastoma cells. *Biochem Pharmacol* 72:19–31
- Ryzhov S, McCaleb JL, Goldstein AE, Biaggioni I, Feoktistov I (2007) Role of adenosine receptors in the regulation of angiogenic factors and neovascularisation in hypoxia. *J Pharmacol Exp Ther* 320:565–572
- Fredholm BB, Ijzerman AP, Jacobson KA, Klotz K-N, Linden J (2001) Nomenclature and classification of adenosine receptors. *Pharmacol Rev* 53:527–552
- Merighi S, Mirandola P, Varani K, Gessi S, Leung E, Baraldi PG et al (2003) A glance at adenosine receptors: novel target for antitumor therapy. *Pharmacol Therapeut* 100:31–48
- Jacobson KA, Gao ZG (2006) Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* 5:247–264
- Gessi S, Merighi S, Sacchetto V, Simioni C, Borea PA (2011) Adenosine receptors and cancer. *Biochim Biophys Acta* 1808:1400–1412
- Pfragner R, Wimsberger G, Niederle B, Behmel A, Rinner I, Mandl A et al (1996) Establishment of a continuous cell line from a human carcinoid of the small intestine (KRJ-I): characterization and effects of 5-azacytidine on proliferation. *Int J Oncol* 8:513–520
- Modlin IM, Kidd M, Pfragner R, Eick GN, Champaneria C (2006) The functional characterization of normal and neoplastic enterochromaffin cells. *J Clin Endocr Metab* 91:2340–2348
- Kidd M, Eick GN, Modlin IM, Pfragner R, Champaneria MC, Murren J (2007) Further delineation of the continuous human neoplastic enterochromaffin cell line, KRJ-I, and the inhibitory effects of lanreotide and rapamycin. *J Mol Endocrinol* 38:181–192
- Rees DA, Lewis MD, Lewis BM, Smith PJ, Scanlon MF, Ham J (2002) Adenosine-regulated cell proliferation in pituitary folliculostellate and endocrine cells: differential roles for the A₁ and A_{2B} adenosine receptors. *Endocrinology* 143:2427–2436
- Merighi S, Benini A, Mirandola P, Gessi S, Varani K, Simioni C et al (2007) Caffeine inhibits adenosine-induced accumulation of HIF-1 α , VEGF and IL-8 expression in hypoxic human colon cancer cells. *Mol Pharmacol* 72:395–406
- Lu J, Pierron A, Ravid K (2003) An adenosine analogue, IB-MECA, down-regulates estrogen receptor alpha and suppresses human breast cancer cell proliferation. *Cancer Res* 63:6413–6423
- Ohana G, Bar-Yehuda S, Arich A, Madi L, Deznick Z, Rath-Wolfson L et al (2003) Inhibition of primary colon carcinoma growth and liver metastasis by the A₃ adenosine receptor agonist CF101. *Brit J Cancer* 89:1552–1558
- Panjehpour M, Karami-Tehrani F (2007) Adenosine modulates cell growth in the human breast cancer cells via adenosine receptors. *Oncol Res* 16:575–585
- Saito M, Yaguchi T, Yasuda Y, Nakano T, Nishizaki T (2010) Adenosine suppresses CW2 human colonic cancer growth by inducing apoptosis via A(1) adenosine receptors. *Cancer Lett* 290:211–215
- Mirza A, Basso A, Black S, Malkowski M, Kwee L, Pachter JA et al (2005) RNA interference targeting of A1 receptor-overexpressing breast carcinoma cells leads to diminished rates of cell proliferation and induction of apoptosis. *Cancer Biol Ther* 4:1355–1360
- Christofi FL, Kim M, Wunderlich JE, Xue J, Suntres Z, Cardounel A et al (2004) Endogenous adenosine differentially modulates 5-hydroxytryptamine release from a human enterochromaffin cell model. *Gastroenterology* 127:188–202
- Spychala J (2000) Tumor-promoting functions of adenosine. *Pharmacol Therapeut* 87:161–173
- Pellegatti P, Raffaghello L, Bianchi G, Piccardi F, Pistoia V, Di Virgilio F (2008) Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. *PLoS One* 3:e2599

29. Stagg J, Smyth MJ (2010) Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene* 29:5346–5358
30. Li S, Huang S, Peng SB (2005) Overexpression of G-protein-coupled receptors in cancer: involvement in tumor progression. *Int J Oncol* 27:1329–1339
31. Panjehpour M, Castro M, Klotz KN (2005) Human breast cancer cell line MDA-MB-231 expresses endogenous A_{2B} adenosine receptors mediating a Ca⁺⁺ signal. *Brit J Pharmacol* 145:211–218
32. Xiang HJ, Liu ZC, Wang DS, Chen Y, Yang YL, Dou KF (2006) Adenosine A_{2B} receptor is highly expressed in human hepatocellular carcinoma. *Hepatol Res* 36:56–60
33. Rodrigues S, de Wever O, Bruyneel E, Rooney RJ, Gespach C (2007) Opposing roles of netrin-1 and the dependence receptor DCC in cancer cell invasion, tumor growth and metastasis. *Oncogene* 26:5615–5625
34. Ryzhov S, Novitskiy SV, Zaynagetdinov R, Goldstein AE, Carbone DP, Biaggioni I et al (2008) Host A(2B) adenosine receptors promote carcinoma growth. *Neoplasia* 10:987–995
35. Ohta A, Gorelik E, Prasad SJ, Ronchese F, Lukashev D, Wong MKK et al (2006) A_{2A} adenosine receptor protects tumors from antitumor T cells. *P Natl Acad Sci USA* 103:13132–13137
36. Lukashev D, Sitkovsky M, Ohta A (2007) From “Hellstrom Paradox” to anti-adensinergic cancer immunotherapy. *Purinerg Signal* 3:129–134
37. Kudlacek O, Just H, Korkhov VM, Vartian N, Klinger M, Pankevych H et al (2003) The human D2 dopamine receptor synergizes with the A_{2A} adenosine receptor to stimulate adenylyl cyclase in PC12 cells. *Neuropsychopharmacol* 28:1317–1327
38. Srirajaskanthan R, Watkins J, Marelli L, Khan K, Caplin ME (2009) Expression of somatostatin and dopamine 2 receptors in neuroendocrine tumours and the potential role for new biotherapies. *Neuroendocrinology* 89:308–314
39. Stagg J, Divisekera U, McLaughlin N, Sharkey J, Pommey S, Denoyer D et al (2010) Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *P Natl Acad Sci USA* 107:1547–1552
40. Etique N, Grillier-Vuissoz I, Lecomte J, Flament S (2009) Crosstalk between adenosine receptor (A_{2A} isoform) and ERalpha mediates ethanol action in MCF-7 breast cancer cells. *Oncol Rep* 21:977–981
41. Mujoomdar M, Hoskin D, Blay J (2003) Adenosine stimulation of the proliferation of colorectal carcinoma cell lines. Roles of cell density and adenosine metabolism. *Biochem Pharmacol* 66:1737–1747
42. Mujoomdar M, Bennett A, Hoskin D, Blay J (2004) Adenosine stimulation of proliferation of breast carcinoma cell lines: evaluation of the [³H]thymidine assay system and modulatory effects of the cellular microenvironment in vitro. *J Cell Physiol* 201:429–438
43. Syversen U, Ramstad H, Gamme K, Qvigstad G, Falkmer S, Waldum HL (2004) Clinical significance of elevated serum chromogranin A levels. *Scand J Gastroentero* 39:969–973
44. Oberg K, Eriksson B (2005) Endocrine tumours of the pancreas. *Best Pract Res Cl Ga* 19:753–781
45. Canaff L, Bevan S, Wheeler DG, Moulant AJ, Rehfuss RP, White JH et al (1998) Analysis of molecular mechanisms controlling neuroendocrine cell specific transcription of the chromogranin A gene. *Endocrinology* 139:1184–1196