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European Journal of Pharmacology 341 (1998) 299–308

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Adenosine modulates cell proliferation in human colonic carcinoma. II. Differential behavior of HT29, DLD-1, Caco-2 and SW403 cell lines

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Received 24 April 1997; revised 21 October 1997; accepted 24 October 1997

Abstract

In a previous study, we provided evidence that extracellular adenosine modulates growth of the poorly differentiated colonic adenocarcinoma cells HT29 and proposed that adenosine A_1 receptors might mediate proliferative effects. We now extend our investigations to a group of colonic adenocarcinoma cells at different stages of enterocytic differentiation. In HT29, DLD-1, Caco-2 and SW403, proliferation was decreased in the presence of adenosine deaminase (5 or 10 U/ml), 5′-*N*-ethylcarboxamido-adenosine (NECA; 1 μM), xanthine amine congener and 8-phenyltheophylline (both at 1 nM or 1 μM). NECA stimulated cAMP accumulation in all cell lines except for HT29. In the presence of forskolin (adenyl cyclase activator) cAMP accumulation was inhibited at sub-nanomolar concentrations of NECA and stimulated at micromolar concentrations in all four cell lines. The inhibitory response disappeared in the presence of 50 nM cyclopentyladenosine (CPA). The binding of [^{3}H]cyclopentyl-1,3-dipropylxanthine and [^{3}H]NECA was also investigated in the four cell lines. Results of displacement experiments were consistent with the idea that poorly differentiated cells with high proliferation rates (e.g. HT29) express mainly adenosine A_1 receptors. In contrast, displacement curves with more differentiated cells exhibiting low proliferation rates (e.g. Caco-2, DLD-1, SW403) displayed two components. The high-affinity component was no longer seen in competition experiments performed in the presence of [^{3}H]NECA and 50 nM CPA. Together, our results further support the idea that extracellular adenosine stimulates cell proliferation in colonic adenocarcinoma cells. The effects might involve cAMP-coupled adenosine receptors. © 1998 Elsevier Science B.V.

Keywords: Adenosine; Colonic adenocarcinoma cell line; Cell growth; Cell differentiation; Adenosine A_1 receptors; Adenosine A_2 receptor

1. Introduction

Adenosine appears to be an essential and ubiquitous nucleoside, taking part in the metabolism of nucleic acids, second messengers, cofactors of methyltransferases and ATP regeneration (Meghji, 1991). Furthermore, adenosine is also a well-known extracellular messenger. This auto/paracrine factor binds cell-surface receptors coupled with the adenylate cyclase: The adenosine A_1 and adenosine A_3 receptor subtypes mediate inhibition, while the A_2 receptor subtype mediates activation of the cyclase. They all belong to the so called 'R7G' (seven transmembrane-spanning domains, G protein-coupled) receptor family (Olah and Stiles, 1995).

There are indications that adenosine may modulate cell proliferation and differentiation in human endothelial, astrocytoma and epidermoid carcinoma cells in culture (Rathbone et al., 1992; Tey et al., 1992; Ethier et al., 1993). Furthermore, adenosine may also affect cell differentiation as observed in neuroblastoma (Abbracchio et al., 1989). We have demonstrated earlier that extracellular adenosine deprivation and treatment with adenosine receptor antagonists reduces the proliferation of human adenocarcinoma HT29 cells. The treatments also resulted in transient elevation of intracellular cAMP levels; in addition, an adenosine A_1-selective receptor agonist and an adenosine A_1-selective receptor antagonist bound specifically to these cells. Thus, it was proposed that endogenous adenosine could participate in the process of HT29 proliferation and that it might mediate its effects, at least partly, through adenosine A_1 receptors (submitted).

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We were interested to find whether these results could be extended to other colonic adenocarcinoma cell lines. We chose Caco-2, DLD-1 and SW403 cell lines because, in contrast to the HT29 cells that display poor enterocytic features under standard culture conditions, DLD-1 and SW403 exhibit an intermediate differentiated phenotype, and Caco-2 cells exhibit a well-differentiated aspect (Zweibaum et al., 1991).

2. Materials and methods

2.1. Chemicals and materials

Cyclopentyl-1,3-dipropyl[2,3-^{3}H]xanthine: [^{3}H]DPCPX (100 Ci/mmol), 5′-*N*-[adenine-2,8-^{3}H]ethylcarboxamidoadenosine: [^{3}H]NECA (25.7 Ci/mmol) and [^{125}I]cAMP radio-immunoassay kit were from NEN Dupont Research Products (Les Ulis/France). Bacitracin, fetal calf serum, glutamax I Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin and trypsine-EDTA came from Gibco^{BRL} (Life Technologies, Eragny/France). Optiphase II scintillant was purchased from EG&G Division Instruments (Evry/France). Adenosine deaminase type VI from calf intestinal mucosa: EC 3.5.4.4 (ADA), bovine serum albumin, 2-chloroadenosine (CLAD), N6-cyclohexyladenosine (CHA), N6-cyclopentyladenosine (CPA), cyclopentyl-1,3-dipropylxanthine (DPCPX), dimethylsulfoxide, 5′-(*N*-ethyl)-carboxamidoadenosine (NECA), forskolin, HEPES, (*R*)N6-phenylisopropyladenosine (*R*-PIA), phenylmethyl sulfonyl fluoride, 8-phenyltheophylline (8-PT) and Triton X100 were from Sigma Chimie (Saint Quentin/France). Trichloracetic acid was from Merck-Clévenot$^{S.A.}$ (Nogent-sur-Marne/France). Xanthine amine congener (XAC) was from RBI (Bioblock Scientific, Illkirch/France).

2.2. Cell culture

Caco-2, DLD-1, HT29 and SW403 cells were routinely cultured in 75 cm^2 culture flasks in Glutamax I DMEM (25 mM glucose, without sodium pyruvate) supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin (respectively, 100 U/ml and 0.1 mg/ml), in a humidified atmosphere of air/CO_2 (95/5%), at 37°C. The culture medium was removed and replaced by fresh medium every 3 days. For subcultures, cells were harvested in trypsin–EDTA solution (respectively, 0.05%/0.53 mM) in Puck's buffer, for 10 min at 37°C.

1 to 3 days before the day of the experiment, cells were seeded at the desired density (see below) on 24-well plates. The culture dishes were previously coated with HT29 extracellular matrix to optimize cell attachment and spreading, according to Bellot et al. (1985). In brief, HT29 cells (0.1×10^6) were seeded in 24-well dishes and cultured until confluency was reached. Cells were then removed by solubilization in 0.3 ml of Triton X100 (0.1%) in phosphate buffered saline (PBS). The wells coated with extracellular matrix were then washed 3 times with sterile PBS. Precoated 24-well plates were kept at −18°C for months.

2.3. Proliferation assays

Dishes were seeded with 10,000 cells in 1 ml of culture medium into 24-well dishes. After 24 h, daily addition (10 μl from a 100 fold concentrated solution) of the specified drugs or of the vehicle was initiated. The vehicle was glycerol (0.3% final concentration) in experiments using adenosine deaminase and dimethylsulfoxide (0.01% final concentration) in experiments using adenosine analogs. The culture medium was replaced every 3 days. At the end of the treatments, the cells were harvested in 500 μl trypsin/EDTA. Protease activity was then stopped by addition of 500 μl culture medium and the number of cells was obtained by double direct counting. Cell viability was evaluated by means of the trypan blue extrusion test. It never exceeded 1%. Cell counts from the supernatant indicated that detachment did not occur.

2.4. Measurements of intracellular cAMP levels in colonic carcinoma cells

Cells seeded at the density of 2×10^6 cells (HT29 and DLD-1) or 3.5×10^6 cells (Caco-2 and SW403), were cultured for 3 days. Then, the culture medium was replaced by 270 μl of fresh medium. Cells were occasionally preincubated for 20 min in the presence of forskolin (5 μM). Treatments with increasing concentrations of the adenosine receptor agonist NECA were then initiated. After 15 min, the plates were placed on ice and the medium was replaced with 300 μl of trichloracetic acid (6% solution). The cells were lysed by direct sonication in the wells and the extracts were evaporated. The resulting pellets were dissolved in sodium acetate buffer (0.05 M, pH = 5.9). After a short centrifugation, the cAMP of the supernatants was quantified using a commercially available cAMP radioimmunoassay, using [^{125}I]cAMP as a tracer and a γ-counter (Wallac; EG&G Instruments, Evry/France).

2.5. Binding of adenosine analogs in colonic carcinoma cells

Cells seeded at the density of 2×10^6 cells (HT29 and DLD-1) or 3.5×10^6 cells (Caco-2 and SW403) in 24-well plates were cultured for 3 days. Then, the cells were incubated for 150 min at 4°C, in 300 μl of binding medium consisting of culture medium containing 15 mM phenylmethyl-sulfonyl-fluoride, 150 μM HEPES, 2 U/ml adenosine deaminase, 0.1% bacitracin, 1% bovine serum albumin (binding buffer) and [^{3}H]DPCPX (5 nM) or

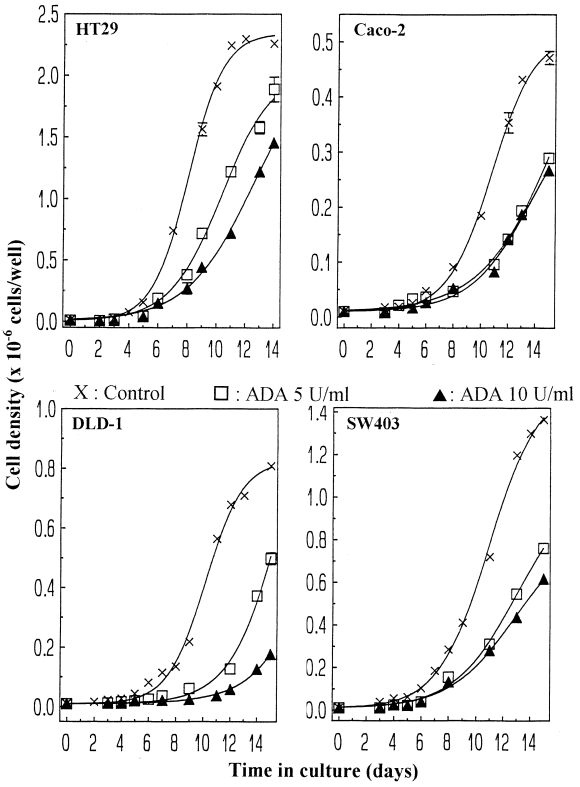

Fig. 1. Effects of adenosine deprivation obtained after daily addition of adenosine deaminase at the concentration of 5 or 10 U/ml. Cells were seeded at 10.000 cells/well in precoated 24-well plates. Culture was ended at the days specified, cells were harvested by trypsinization and counted. Mean ± S.E.M. ($n = 9$) from 3 independent experiments (see Section 2). ×: Control, □: ADA 5 U/ml and ▲: ADA 10 U/ml.

[^{3}H]NECA (10 nM). Conditions were dictated by results of preliminary experiments including time-course studies (data not shown). Non-specific binding was determined in the presence of an excess (5 μM) of the appropriate unlabelled ligand. In addition, because NECA binds to both adenosine A_1 and A_2 receptors, the binding of [^{3}H]NECA was also performed in the presence or in the absence of 50 nM of CPA in order to prevent binding on the adenosine A_1 receptor subtypes, as previously described by others (Blazynski and McIntoch, 1993). [^{3}H]DPCPX and [^{3}H]NECA binding were competitively displaced by increasing concentrations of adenosine analogs (in the range of 10 pM to 1 μM), in order to determine specificity.

At the end of the incubations, the wells were quickly washed once with 1 ml cold PBS (pH = 7.2). The cells were lysed with 400 μl NaOH (0.5 M) for 30 min and the extracts were incubated overnight in 5 ml of Optiphase II scintillation reagent in counting vials. Radioactivity was measured in a beta scintillation counter (Beckman Instruments, Gagny/France).

2.6. Statistical analysis and curve plots

All the data shown represent the means of 2 or 3 independent experiments. Each experiment involved triplicate determinations. Each determination used double counting in the proliferation experiments. All data were computed using the GRAPHPAD™ (ISI software) iterative curve-fitting program. Data from the proliferation and

Table 1
Growth parameters (latency and doubling time (DT) expressed in hours) from proliferation studies of four colonic carcinoma cell lines in culture in the presence or absence of adenosine deaminase (ADA), xanthine amine congener (XAC), 8-phenyltheophylline (8-PT) or 5′-(*N*-ethyl)-carboxamidoadenosine (NECA) as indicated

	Caco-2		DLD-1		SW403		HT29	
Treatment	Latency	DT	Latency	DT	Latency	DT	Latency	DT
Control	96	44	72	41	43	36	26	25
ADA, 10 U/ml	134^c *C*	66^c *A*	168^c *C*	69^c *C*	85^c *C*	72^c *C*	52^c *C*	60^c *C*
ADA, 5 U/ml	122^c	64^c	144^c	52^c	72^c	58^c	36^c	35^c
XAC, 1 μM	82^c *C*	44 *B*	68^a *B*	44^b *C*	45^a *C*	32^b *C*	39^c *C*	52^c *C*
XAC, 1 nM	103^b	48^b	74^a	54^c	45^a	38^a	25	39^c
8-PT, 1 μM	120^c *A*	64^c *A*	89^c *A*	52^c *B*	51^c *B*	48^c *B*	40^c *C*	46^c *C*
8-PT, 1 nM	122^c	62^c	92^c	48^c	56^c	52^c	32^b	32^b
NECA, 1 μM	134^c	51^b	82^c	46^b	64^c	64^c	42^c	36^c

The two parameters were calculated from data as shown in Fig. 1. Statistical analysis was performed as described in Section 2. Values were compared to their respective control ($^aP < 0.05$, $^bP < 0.002$ or $^cP < 0.00005$), or between the low and the high concentrations of a similar treatment (*A*: $P < 0.05$, *B*: $P < 0.002$ or *C*: $P < 0.00005$). At an equivalent concentration, the values obtained with XAC and 8-PT also differed significantly ($P < 0.005$) in all cases except in HT29 at 1 μM.

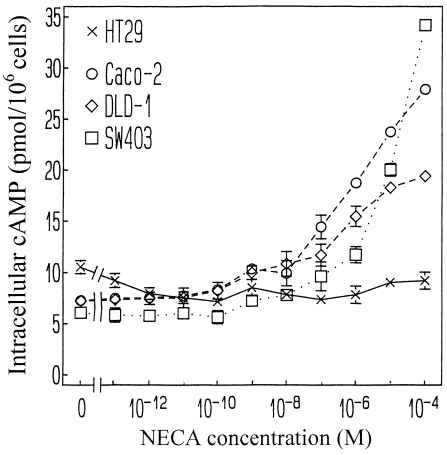

Fig. 2. Intracellular cAMP production in colonic carcinoma cells (×) HT29, (○) Caco-2, (◇) DLD-1 and (□) SW403, in the presence of increasing concentrations of 5′-(*N*-ethyl)-carboxamidoadenosine (NECA). Treatments were run for 15 min at 37°C. Data represent the means ± S.E.M. of 2 independent experiments each performed in triplicate ($n = 6$).

displacement experiments were fitted to a Hill equation. For fitting more complex competitive inhibitions, as observed for [^{3}H]DPCPX or [^{3}H]NECA displacements, the two-site competitive binding curve equation was used. The data are described as a better fit by one model of ligand binding than another when a partial *F*-test comparing the two models indicated significant improvement in residual sum of squares as described previously (Wells, 1992). The *F*-value was calculated according to the equation $F = [(SS1 - SS2)/(df1 - df2)]/(SS2/df2)$, where SS1 and SS2 are the residual sums of squares, with the corresponding degrees of freedom df1 and df2 associated with the simpler and more complex model, respectively.

In the proliferation experiments, latency was defined as the time necessary to reach the first doubling of the population. Values within this range were compared using an analysis of variance (ANOVA). To compare the doubling time values, proliferation curve were linearized (Log[cell number] = $f(t)$) before running the Anova.

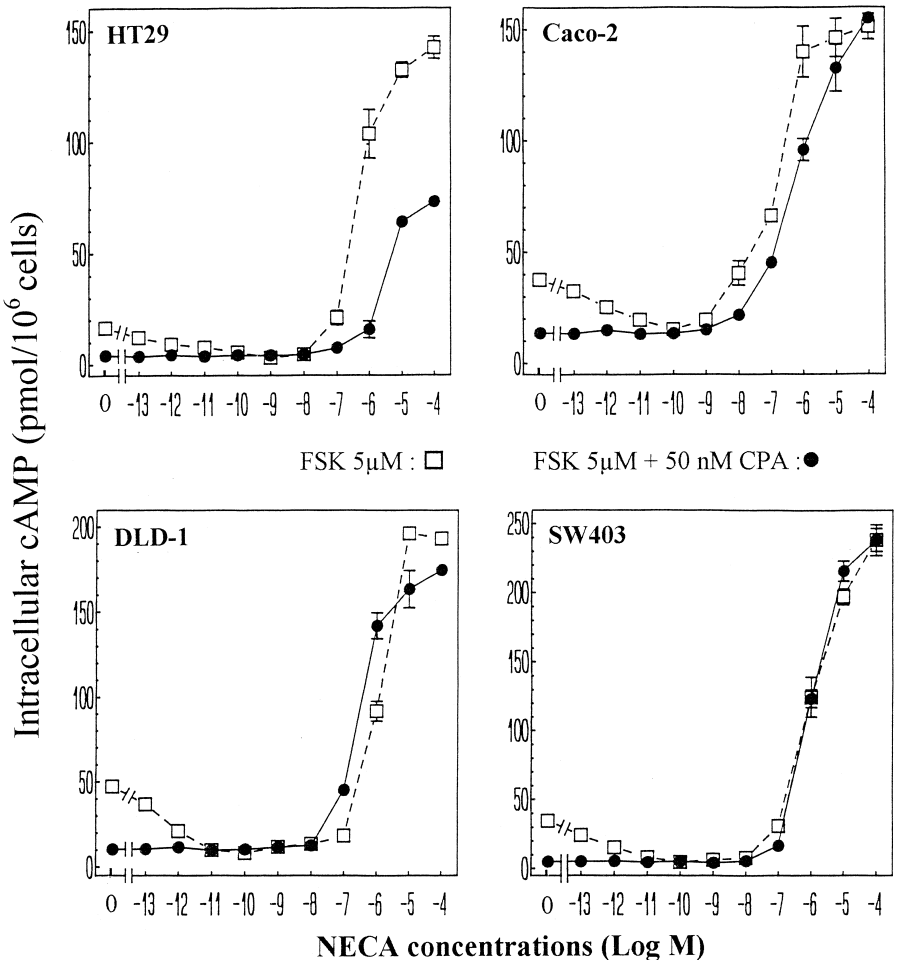

Fig. 3. Intracellular cAMP production in colonic carcinoma cells HT29, Caco-2, DLD-1 and SW403, in response to 15 min stimulation with increasing concentrations of 5′-(*N*-ethyl)-carboxamidoadenosine (NECA; from 0.1 pM to 100 μM) either alone (□) or in the presence (●) of 50 nM N6-cyclopentyladenosine (CPA). Treatments were run for 15 min at 37°C, after 20-min preincubation with 5 μM Forkolin. Data represent the means ± S.E.M. of 2 independent experiments each performed in triplicate ($n = 6$).

3. Results

3.1. Foreword

For easier reading of the present section and to facilitate comparisons of the four cell lines investigated, some of the data of Figs. 6(A) and (B) from previous work (Lelièvre et al., 1998) are shown here.

3.2. Proliferation

Under standard conditions, HT29, Caco-2, DLD-1 and SW403 cells all showed a typical sigmoid growth curve (Fig. 1). Latency and doubling time of each cell line are given in Table 1. The confluency state was reached for a very low density in Caco-2 cells, for an intermediate density in DLD-1 and SW403 cells and for a high density in HT29 cells. In the presence of adenosine deaminase, there was a clear reduction of cell growth in all 4 cell lines (Fig. 1 and Table 1). The effects were stronger with 10 U/ml than with 5 U/ml in HT29, DLD-1 and SW403 cells. In Caco-2 cells these two different concentrations had similar effects (Table 1). The effects of the adenosine antagonists, xanthine amine congener (selective for the adenosine A_1 receptor) and 8-phenyltheophylline (non selective) were also investigated. Data from the growth curves (not shown) allowed us to determine the latency and doubling time (Table 1). At the concentrations of 1 nM and 1 μM, 8-phenyltheophylline induced a reduction in cell growth in all four cell lines. The amplitude of the inhibition induced by each concentration was more or less similar in Caco-2 and DLD-1, respectively. The higher dose induced smaller effects than the lower dose in SW403, whereas the opposite held true for HT29 (Table 1). At the concentration of 1 nM, xanthine amine congener induced inhibition of cell growth in the four cell lines. At the concentration of 1 μM clear cut inhibitory effects were observed in HT29 cells. In Caco-2 and DLD-1 latency was even decreased. In Caco-2, DLD-1 and SW403 cells, the inhibitions induced by 8-phenyltheophylline were higher

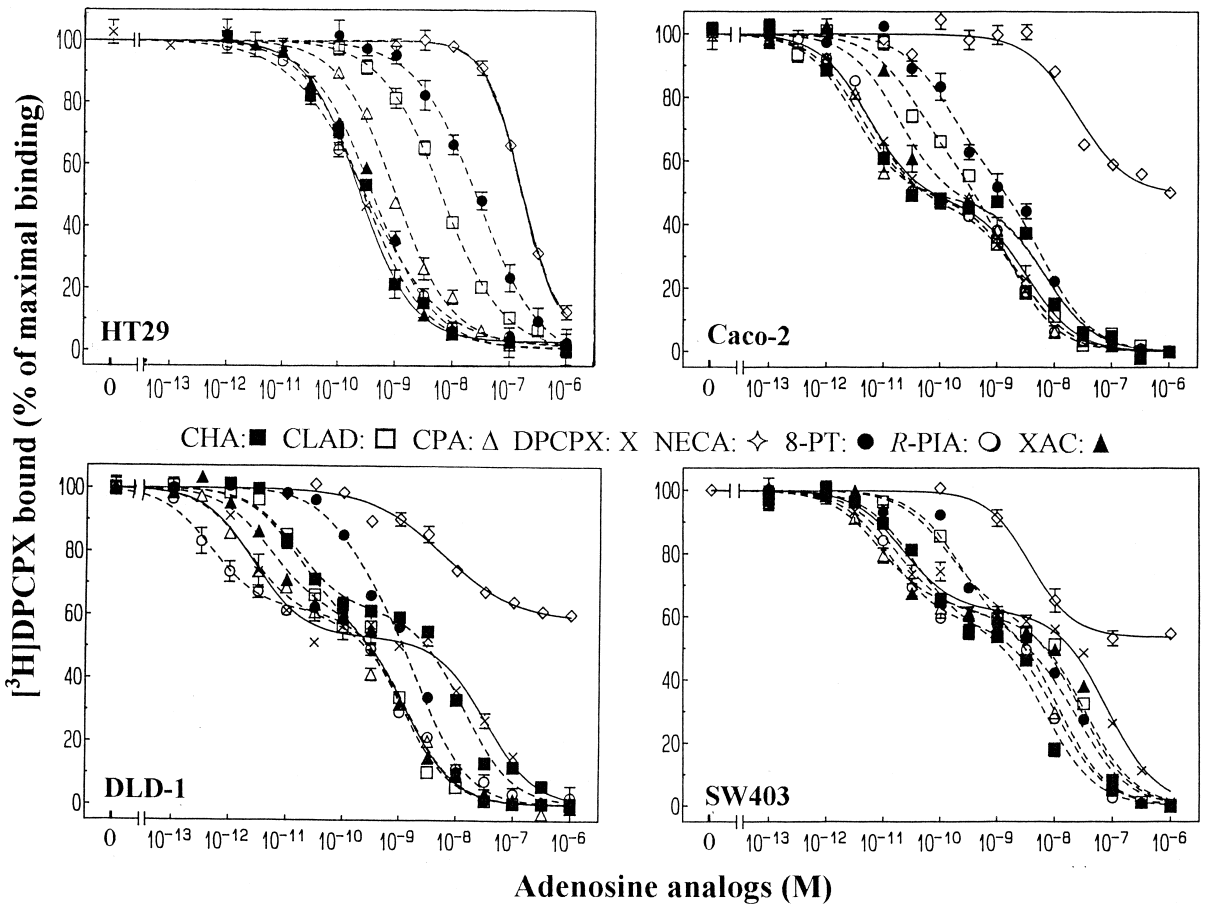

Fig. 4. Competitive inhibition of the binding of [^{3}H]DPCPX (5 nM) on intact carcinoma HT29, Caco-2, DLD-1 and SW403 cells, by the adenosine analogs CHA (N6-cyclohexyladenosine, ■), CLAD (2-chloroadenosine, □), CPA (N6-cyclopentyladenosine, △), DPCPX (cyclopentyl-1,3-dipropylxanthine, X), NECA (5′-(*N*-ethyl)-carboxamidoadenosine, ◇), 8-PT (8-phenyltheophylline, ●), R-PIA ((*R*)N6-phenylisopropyladenosine, ○) or XAC (xanthine amine congener, ▲). Cells were incubated for 150 min at 4°C in the presence of the radioligand and the specified analogs at the concentrations indicated. Data represent the means ± S.E.M. of 3 independent experiments each performed in triplicates ($n = 9$). A partial *F*-test indicated that the Hill equation was the best fit for HT29 cells, whereas the 2-sites equation was the most appropriate for Caco-2, DLD-1 and SW403.

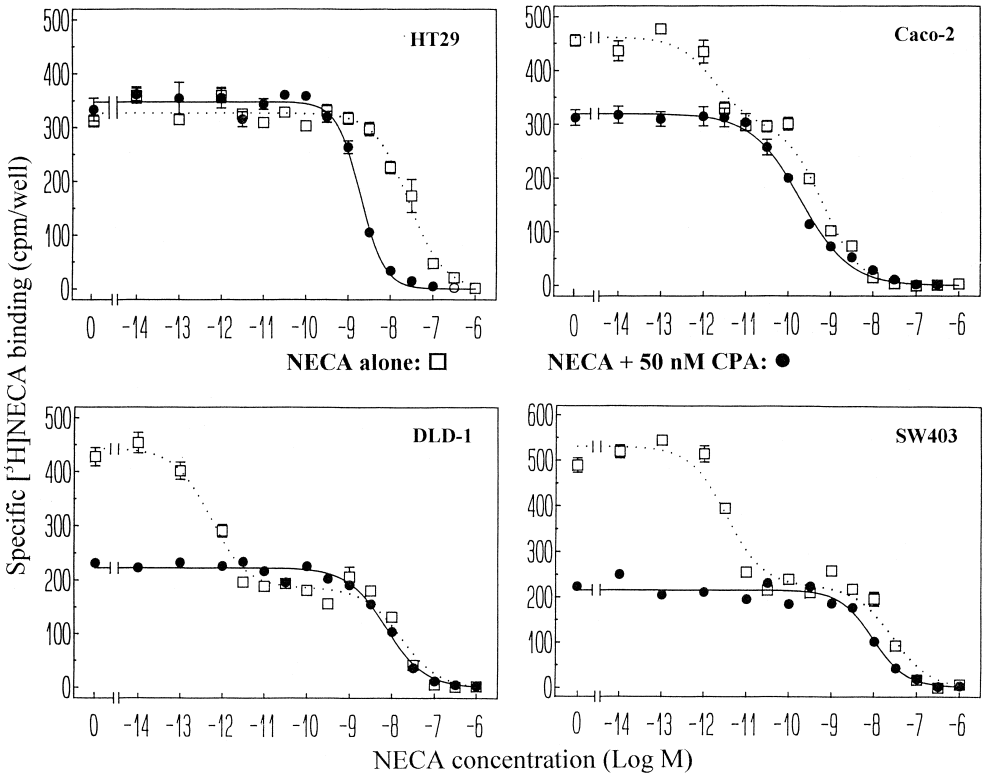

Fig. 5. Competitive inhibition of the binding of [^{3}H]NECA ([^{3}H]5′-(*N*-ethyl)-carboxamidoadenosine; 10 nM) on intact carcinoma HT29, Caco2, DLD-1 and SW403 cells, by increasing concentrations of NECA either alone (□) or in the presence of 50 nM N6-cyclopentyladenosine (●) (CPA; adenosine A_1-preferring receptor agonist). Incubations were run for 150 min at 4°C. Data represent the means ± S.E.M. of 3 independent experiments each performed in triplicate ($n = 9$). A partial *F*-test indicated that the Hill equation was the best fit for HT29 cells, whereas the 2-site equation was the most appropriate for Caco-2, DLD-1 and SW403, but in the absence of CPA only.

than those triggered by xanthine amine congener (Table 1). The adenosine receptor agonist, NECA (1 μM), induced an increase in latency and doubling time in all cell lines.

3.3. Cyclic AMP

The effects of increasing concentrations of NECA, a non-selective agonist (from 1 pM to 100 μM) were investigated at 15 min of treatment. This incubation time was dictated by results of previous time course experiments (data not shown) which showed that NECA (1 μM) induced maximal effects at 15 ± 3 min. In Caco-2, DLD-1 and SW403 cells, NECA induced a dose-dependent increase in cAMP content (Fig. 2). No effect was detected in HT29 cells. (Fig. 2).

Table 2
Displacement of the [^{3}H]DPCPX binding in Caco-2, DLD-1, HT29 and SW403 cell lines, by unlabelled analogs

Adenosine analogs	HT29		Caco-2		DLD-1		SW403	
	site	Hill	site 1	site 2	site 1	site 2	site 1	site 2
DPCPX	0.2 ± 0.084	0.62	5.5 ± 0.05	3.3 ± 0.055	2.7 ± 0.098	3 ± 0.13	20 ± 0.12	81 ± 0.21
CHA	0.28 ± 0.056	0.81	4.8 ± 0.18	2.9 ± 0.17	15 ± 0.10	13 ± 0.17	33 ± 0.098	7 ± 0.085
CLAD	7.4 ± 0.075	0.73	37 ± 0.08	1.5 ± 0.09	14 ± 0.09	1.15 ± 0.135	145 ± 0.24	28 ± 0.145
CPA	0.95 ± 0.071	0.87	4 ± 0.15	2.5 ± 0.12	2.1 ± 0.1	1.35 ± 0.14	9 ± 0.045	11 ± 0.15
NECA	152 ± 0.096	0.94	none	20 ± 0.045	none	5.7 ± 0.048	none	3.7 ± 0.08
8-PT	46 ± 0.077	0.85	130 ± 0.09	6 ± 0.08	147 ± 0.14	2.4 ± 0.045	230 ± 0.22	27 ± 0.22
PIA	0.3 ± 0.082	0.71	5.7 ± 0.095	2.6 ± 0.13	0.48 ± 0.07	1.3 ± 0.14	13.9 ± 0.145	10 ± 0.29
XAC	0.46 ± 0.056	0.89	18 ± 0.08	7 ± 0.09	4.9 ± 0.09	1.2 ± 0.078	18 ± 0.13	33 ± 0.33
site 1/site 2 (%)	100		50	50	40	60	45	55

All values (including means ± S.E.M.) were obtained from data shown in Fig. 4. CHA: N6-cyclohexyladenosine, CLAD: 2-chloroadenosine, CPA: N6-cyclo-pentyladenosine, DPCPX: cyclopentyl-1,3-dipropylxanthine, NECA: 5′-(*N*-ethyl)-carboxamidoadenosine, *R*-PIA: (*R*)N6-phenylisopropyladenosine, 8-PT: 8-phenyltheophylline, XAC: xanthine amine congener.

Similar experiments were run in the presence of the adenylate cyclase activator, forskolin (5 μM) (Fig. 3, open squares). In all 4 cell lines, forskolin induced an increase in cAMP accumulation (compare basal values in Fig. 2, with basal values in Fig. 3, open squares). With concentrations ranging from 0.01 to 1 nM, NECA induced a dose-dependent decrease in cAMP content. A dramatic dose-dependent increase was observed with concentrations over 10 nM. In another series of experiments, the cells were cultured in the presence of both forskolin (5 μM) and the adenosine A_1 receptor agonist CPA (50 nM) (Fig. 3, full circles). Under these conditions, (1) basal cAMP levels were decreased when compared to those observed in the absence of CPA and (2) the inhibitory effect of NECA was no longer observed (Fig. 3). In addition, in HT29 cells the NECA-induced accumulation of cAMP was less in the presence than in the absence of the adenosine analog (Fig. 3).

3.4. Binding of adenosine analogs

The ability of adenosine analogs to displace [^{3}H]DPCPX (5 nM) and [^{3}H]NECA (10 nM) was studied in Caco-2, DLD-1, HT29 and SW403 cells (Figs. 4 and 5). The binding conditions (tritiated analog concentrations, incubation times, etc.) were dictated by results of preliminary experiments (not shown here). There were no major differences in the kinetic parameters in the four cell lines investigated. The data fitted better the one-site competitive inhibition equation (Hill curve) for HT29 cells and a two-site competitive inhibition equation for SW403, DLD-1 and Caco-2 cells. In these 3 cell lines, NECA was unable to trigger complete inhibition of [^{3}H]DPCPX binding. Pharmacological parameters of [^{3}H]DPCPX binding are summarized in Table 2. The following pharmacological profiles were obtained:

HT29 cells:

DPCPX > CHA ≈ R-PIA > XAC > CPA ≫ CLAD ≫ 8-PT ≫ NECA

SW403 cells:

site 1: CPA > R-PIA > XAC ≈ DPCPX > CHA ≫ CLAD ≫ 8-PT

site 2: NECA > CHA > PIA ≈ CPA > CLAD ≈ 8-PT > XAC ≫ DPCPX

DLD1 cells:

site 1: R-PIA ≫ CPA ≈ DPCPX > XAC > CLAD ≈ CHA ≫ 8-PT

site 2: CLAD > XAC ≈ R-PIA ≈ CPA > 8-PT > DPCPX > NECA > CHA

Caco-2 cells:

site 1: CPA > CHA > DPCPX ≈ R-PIA > XAC > CLAD > 8-PT

site 2: CLAD > CPA ≈ R-PIA ≈ CHA ≈ DPCPX > 8-PT > XAC ≫ NECA

Table 3
Displacement of [^{3}H]NECA (5′-(*N*-ethyl)-carboxamidoadenosine; 10 nM) binding by unlabelled NECA either in the presence or in the absence of 50 nM CPA (cyclopentyladenosine)

[^{3}H]NECA	IC_{50}	HT29	Caco-2	DLD-1	SW403
Alone	site 1 (pM)	none	1.8 ± 0.09	0.5 ± 0.11	3 ± 0.12
	site 2 (nM)	25 ± 1.23	0.7 ± 0.055	0.16 ± 0.09	30 ± 0.15
	Hill Coef.	0.85			
+50nM CPA	site (nM)	4 ± 1.14	0.9 ± 0.85	5 ± 0.21	7 ± 0.15
	Hill coef.	0.92	0.76	0.82	0.85
	% Site 1	—	40	50	60
	% Site 2	100	60	50	40

Values (including means ± S.E.M.) were calculated from data shown in Fig. 5.

Dose-dependent competitive inhibition of [^{3}H]NECA binding was evaluated using unlabelled NECA concentrations from 10 pM to 1 μM. In Fig. 5, the data were plotted using a one-site competitive inhibition equation for HT29 cells, while for Caco-2, SW403 and DLD-1 cells, the best fit was obtained with the two-site equation. When the experiments were performed in the presence of 50 nM CPA, no change was observed for HT29 cells. However, a shift from a two-site to a one-site model was recorded for the other cells. The data are reported in Table 3.

4. Discussion

Only few studies, with rather controversial results, have previously demonstrated that adenosine may be involved as a regulator of cell growth. Some of them demonstrated a direct effect of adenosine through pyrimidine nucleotide synthesis or cell apoptosis (Ishii and Green, 1973; Tanaka et al., 1994; Abbracchio et al., 1995); others proposed that adenosine-induced growth modulation was mediated through cell surface receptors (Jackson and Carlson, 1992; Tey et al., 1992; Ethier et al., 1993; Dubey et al., 1996). We have shown (Lelièvre et al., 1998) that endogenous adenosine modulates cell proliferation in HT29 cells and have proposed that these effects might involve, at least in part, adenosine A_1 receptors.

We now demonstrated that adenosine deaminase, which depletes endogenous extracellular adenosine, reduced cell proliferation in the 3 colonic adenocarcinoma cell lines Caco-2, DLD-1 and SW403, as well as in HT29. This supports the idea that extracellular adenosine participates actively in the control of growth in colon cancer cells. Adenosine deaminase-induced inhibition of cell proliferation resulted in both a delay of latency and an increase of the doubling time. In the present study, 2 doses of adenosine deaminase were used. It seemed that HT29 and DLD-1 cells were able to discriminate between the two concentrations better than did SW403 and Caco-2. This suggests that extracellular adenosine levels might differ from one cell line to another.

In all 4 cell lines, the non-permeant adenosine antagonists, xanthine amine congener and 8-phenyltheophylline were able to mimick the effects of adenosine deprivation, as previously shown for HT29 cells (Lelièvre et al., 1998). This strongly supports the hypothesis that endogenous adenosine was acting through cell surface receptors to modulate proliferation (see below). Thus, the above-discussed effects of adenosine deaminase most probably resulted from removal of adenosine, rather than from production of inosine. Regarding the effects of the antagonists, it is noteworthy that HT29 behaved differently from the other 3 cell lines. Indeed, at the concentrations tested, xanthine amine congener was more potent than 8-phenyltheophylline in HT29 cells, whereas this did not hold true for Caco-2, DLD-1 and SW403. Xanthine amine congener displays a high affinity and selectivity for adenosine A_1 receptor subtypes, whereas 8-phenyltheophylline is a non selective adenosine receptor antagonist. The above-mentioned differences among the four cell lines might reflect differences in the distribution of the adenosine receptors and/or in the availability of extracellular adenosine. We have shown that adenosine A_1 receptors are highly expressed in HT29 cells, and suggested that they might mediate the stimulatory effects of extracellular adenosine on cell growth (Lelièvre et al., 1998). A possible explanation would be that HT29 cells express a high proportion of adenosine A1 receptors versus other adenosine receptor subtypes, whereas the other cell lines express at least two classes of receptors mediating antagonistic effects on cell growth. This hypothesis gains support from the observation that xanthine amine congener-induced cell growth inhibition was observed (1) in all cell lines at the nanomolar concentration which antagonizes specifically the adenosine A_1 receptors and (2) only in HT29, at the micromolar concentration where the compound displays little (if any) selectivity. These results fit well with the hypothesis according to which the inhibitory effects would be maintained when a single class of receptors is affected (e.g. HT29) and not when several classes displaying antagonistic properties on cell growth are present (e.g. Caco-2, DLD-1, SW403).

The observation that the non-selective adenosine receptor agonist, NECA, induced inhibitory effects similar to those of the antagonists, supports the view that adenosine A_2 receptors might also be involved which would mediate inhibition of cell growth. Indeed, at the concentration of 1 μM, NECA could bind both adenosine A_1 and A_2 receptors. Interestingly, NECA induced stimulation of cAMP content in all cell lines except HT29. This would suggest that either adenosine A_2 receptors are present in Caco-2, DLD-1 and SW403 and absent in HT29 cells, or that the adenosine A_1/adenosine A_2 receptor ratio is different in these cell lines. As a matter of fact, an A_2-like response to NECA could be evidenced in HT29 cells only in the presence of the adenylate cyclase activator forskolin. As already proposed for other cellular systems (Seamon and Daly, 1986), in HT29 cells, Gs-protein mobilization by adenosine A_1 receptors might have synergistically potentiated the effects of forskolin on the catalytic subunit of adenylate cyclase. In addition to the increase in basal cAMP levels, forskolin revealed an inhibitory response at low concentrations of NECA, in all four cell lines. This bimodal modulation of cAMP levels by NECA has been observed in the bovine retina by others (Blazynski and McIntoch, 1993). It suggests that both high-affinity adenosine A_1 receptors and low-affinity adenosine A_2 receptors were expressed in the cancer cells now studied. The results of experiments performed in the presence of 50 nM of CPA (an adenosine A_1-preferring receptor agonist) further support this view. Indeed, in the presence of the adenosine A_1 receptor agonist CPA, control cAMP levels were decreased and the inhibitory response to NECA was no longer observed, in agreement with the idea that accessibility of NECA to adenosine A_1 receptors was suppressed. In HT29 cells, such a combined treatment also induced a noticeable reduction of the stimulatory response. This would agree with the idea that adenosine A_2 receptors might have been partially blocked by CPA in this cell line.

As proposed elsewhere (Cho-Chung, 1990), a positive relationship might exist between cAMP levels and cell growth inhibition in Caco-2, DLD-1, SW403 and HT29 cells. Indeed, adenosine deaminase or adenosine receptor antagonists (xanthine amine congener or 8-phenyltheophylline), which induced a dramatic elevation of cAMP content in HT29 cells (Lelièvre et al., 1998), also induced inhibition of cell growth in all four cell lines. Moreover, NECA also induced cell growth inhibition at a concentration which elevates cAMP levels. It may be hypothesized that endogenous adenosine acts mainly through adenosine A_1 receptors to maintain low cAMP levels in HT29 cells, whereas it acts on both A_1 and A_2 receptors in the other three cell lines. The binding results support this view.

The presence of adenosine A_1 binding sites has been reported in HT29 (Lelièvre et al., 1998). We now extended these findings to Caco-2, DLD-1 and SW403. However, whereas data from the displacement experiments fitted the Hill equation for HT29 cells, two components were evidenced in Caco-2, DLD-1 and SW403. The order of potency of the unlabelled analogs was typical of that observed for adenosine A_1 binding sites in the high-affinity range. This was not reproduced in the low affinity range. Interestingly, NECA (in the range of 10 pM to 1 μM) triggered only partial displacement of [^{3}H]DPCPX. The two components might reflect the presence of either adenosine A_1 receptors displaying different affinity states (for extensive discusssion see: Marteens et al., 1988; Ramkumar et al., 1988; Cohen et al., 1996), or two categories of binding sites. The second hypothesis is supported by the following observations: (1) Hill coefficients of 0.8 and below were obtained when data were fitted to the Hill equation, suggesting the presence of more than one site (Limbird, 1986), (2) displacement of [^{3}H]NECA

binding by NECA was resolved by an equation of the two-site model for Caco-2, DLD-1 and SW403 cells, but the high-affinity component could not be observed in the presence of 50 nM CPA, a concentration which inactivates adenosine A_1 receptors, (3) also, NECA was able to modulate cAMP levels in both directions in all 4 cell lines, but only stimulation was observed after addition of 50 nM CPA and (4) preliminary binding experiments were performed using [^{3}H]CGS21680, a compound highly specific for the adenosine A_{2A} receptor. The results obtained were similar to those obtained with [^{3}H]NECA. The presence of adenosine A_2 receptors would not be surprising in colon adenocarcinomas. Adenosine A_{2B} receptors have been detected in relative abundance in the gastrointestinal tract (Ongini and Fredholm, 1996). Future experiments will aim to determine the exact nature (A_{2A}, A_{2B}) of the adenosine binding sites so evidenced.

In conclusion, colonic carcinoma cell proliferation appears to be modulated by endogenous adenosine. The results obtained from different experimental approaches lead to the suggestion that (1) the nucleoside might act through adenosine A_1 receptors to lower cAMP levels, a situation which would favor cell proliferation, (2) adenosine A_2 receptors are also present and might be mediating opposite effects. The differences observed between the four cell lines now studied may be related to their state of differentiation. We suggest that over-expression of adenosine A_1 receptors is associated with exacerbated proliferation and poor differentiation, as seen in HT29 cells. This would be consistent with the observation that adenosine A_1 receptor mRNA over-expression occurs in colonic mucosa along with the tumorigenic process, when compared to normal mucosa (Khoo et al., 1996). Such a situation would not occur in cell lines with higher differentiation and low proliferation rates such as Caco-2, DLD-1 and SW403, where the balance between the adenosine A_1 and adenosine A_2 receptors would not be so different. Interestingly, after treating HT29 cells for one month with adenosine deaminase (10 U/ml) we were able to obtained sub-clones, some of which resemble Caco-2 cells more than the mother clone with regard to cell ultrastructure, cAMP response to adenosine analogs, binding of [^{3}H]DPCPX and [^{3}H]NECA (in preparation). Future experiments will aim to determine (1) the exact role adenosine A_1 and A_2 receptors play in adenocarcinoma cell growth and (2) whether such a deregulated balance in the A_1/A_2 receptor ratio might be a cause or a consequence of tumorigenesis. In this respect, the numerous synthetic adenosine analogs appear to have a potential as therapeutic substances to be considered in colon tumorigenesis (Stone et al., 1995).

Acknowledgements

This work was supported by the University of Poitiers, the CNRS and the 'Fondation Langlois' (Rennes, France). We are very grateful to F. Chevalier for her devoted and gentille technical assistance.

References

Abbracchio, M.P., Cattabeni, F., Clementi, F., Sher, E., 1989. Adenosine receptors linked to adenylate cyclase activity in human neuroblastoma cells: Modulation during cell differentiation. Neuroscience 30, 819–825.

Abbracchio, M.P., Ceruti, S., Barbieri, D., Franceschi, C., Marloni, W., Biondo, L., Burnstock, G., Cattabeni, F., 1995. A novel action for adenosine: Apoptosis of astroglial cells in rat brain primary cultures. Biochem. Biophys. Res. Commun. 213, 908–915.

Bellot, F., Pichon, J., Luis, J., El Battari, A., Secchi, J., Cau, P., Marvaldi, J., 1985. Extracellular material secreted by human colonic adenocarcinoma cell lines (HT29 and HRT18) promotes their own very rapid spreading in serum free medium and induces neurite outgrowth of PC 12 cells. Int. J. Cancer 36, 609–615.

Blazynski, C., Mc Intoch, H., 1993. Characterization of adenosine A_2 receptors in bovine retinal membranes. Exp. Eye Res. 56, 585–593.

Cho-Chung, Y.S., 1990. Role of cyclic AMP receptor proteins in growth, differentiation and suppression of malignancy: New approaches to therapy. Cancer Res. 50, 7093–7100.

Cohen, F.R., Lazareno, S., Birdsall, N.J.M., 1996. the effects of saponin on the binding and functional properties of the human adenosine A_1 receptor. Br. J. Pharmacol. 117, 1521–1529.

Dubey, R.K., Gillespie, D.G., Osaka, K., Suzuki, F., Jackson, E.K., 1996. Adenosine inhibits growth of rat aortic smooth muscle cells. Hypertension 27, 786–793.

Ethier, M.F., Chander, V., Dobson, J.G. Jr., 1993. Adenosine stimulates proliferation of human endothelial cells in culture. Am. J. Physiol. 265, H131–138.

Ishii, K., Green, H., 1973. Lethality of adenosine for cultured mammalian cells by interference with pyrimidine biosynthesis. J. Cell. Sci. 13, 429–439.

Jackson, J.A., Carlson, E.C., 1992. Inhibition of bovine retinal microvascular pericyte proliferation in vitro by adenosine. Am. J. Physiol. 263, H634–H640.

Khoo, H.-E., Ho, C.-L., Chhatwal, V.J.S., Chan, S.T.F., Ngoi, S.-S., Moochhala, S.M., 1996. Differential expression of adenosine A_1 receptors in colorectal cancer and related mucosa. Cancer Lett. 106, 17–21.

Lelièvre, V., Muller, J.N., Falcón, J., 1998. Adenosine modulates cell proliferation in human colonic adenocarcinoma. I. Possible involvement of adenosine A_1 receptor subtypes in HT29 cells. Europ. J. Pharmacol., this issue.

Limbird, L.E., 1986. Cell Surface Receptors: A Short Course on Theory and Methods. Martnus-Nijhoff, Boston.

Marteens, D., Lohse, M.J., Schwabe, U., 1988. [^{3}H]8-cyclopentyl-1-3-dipropylxanthine binding to A_1 adenosine receptors of intact Rat ventricular myocytes. Circ. Res. 63, 613–620.

Meghji, P., 1991. Adenosine production and metabolism. In: Stone, T. (Ed.), Adenosine in the Nervous System. Academic Press, London, pp. 25–42.

Olah, M.E., Stiles, G.L., 1995. Adenosine receptor subtypes: Characterization and therapeutic regulation. Annu. Rev. Pharmacol. Toxicol. 35, 581–606.

Ongini, E., Fredholm, B.B., 1996. Pharmacology of adenosine A_{2A} receptors. Trends Pharmacol. Sci. 17 (10), 364–372.

Ramkumar, V., Bumgarner, J.R., Jacobson, K.A., Stiles, G.L., 1988. Multiple components of A_1 adenosine receptor-adenylate-cyclase system are regulated in rat cerebral cortex by chronic caffeine ingestion. J. Clin. Invest. 82, 242–247.

Rathbone, M.P., Middlemiss, P.J., Kim, J.-K., Gysbers, J.W., DeForge, S.P., Smith, R.W., Hughes, D.W., 1992. Adenosine and its nu-

cleotides stimulate proliferation of chick astrocytes and human astrocytoma cells. Neurosci. Res. 13, 1–17.

Seamon, K.B., Daly, J.W., 1986. Forskolin: Its biological and chemical properties. Adv. Cyclic Nucleotide Protein Phosphorylation Res. 20, 1–150.

Stone, T.W., Collis, M.G., Williams, M., Miller, L.P., Karasawa, A., Hillaire-Buys, D., 1995. Adenosine: Some therapeutic applications and prospects. In: Cuello, A.C., Collier, B. (Eds.), Pharmacological Sciences: Perspectives for Research and Therapy in the late 1990's. Birkauser Verlag, Basel, pp. 304–309.

Tanaka, Y., Yoshihara, K., Tsuyuki, M., Kamiya, T., 1994. Apoptosis induced by adenosine in human leukemia HL-60 cells. Exp. Cell Res. 213, 242–252.

Tey, H.B., Khoo, H.E., Tan, C.H., 1992. Adenosine modulates cell growth in human epidermoid carcinoma (A431) cells. Biochem. Biophys. Res. Commun. 187, 1486–1492.

Wells, J.W., 1992. Analysis and interpretation of binding at equilibrium. In: Hulme, E.C. (Ed.), Receptor–ligand Interactions, a Practical Approach. IRL press, Oxford, pp. 289–395.

Zweibaum, A., Laburthe, M., Grasset, E., Louvard, D., 1991. Use of cultured cell lines in studies of intestinal cell differentiation and function. In: Field, M., Frizzel, R.A. (Eds.), Handbook of Physiology: The Gastrointestinal System, Intestinal Absorption and Secretion, vol. 4. American Physiological Society, Washington DC, pp. 223–255.